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THE UNIVERSITY OF ALBERTA

STUDIES ON CHICKEN BLOOD COAGULATION

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF MASTER OF SCIENCE

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

by

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UNIVERSITY OF ALBERTA  
FACULTY OF GRADUATE STUDIES

The undersigned certify that they  
have read and recommend to the Faculty of  
Graduate Studies for acceptance, a thesis  
entitled "STUDIES ON CHICKEN BLOOD COAGUL-  
ATION", submitted by C.H. Bigland, D.V.M.,  
in partial fulfilment of the requirements  
for the degree of Master of Science.



## ABSTRACT

Evidence is presented that the blood coagulation mechanism of the adult hen differs from that of the mammalian species by relying on tissue thromboplastin rather than plasma thromboplastin for hemostasis.

The available literature, dealing in whole or in part with avian blood coagulation, is reviewed. Modification of mammalian techniques for chicken blood studies including preparation of coagulation materials and test procedures are outlined.

The mean clotting time of blood from 37 laying hens was found to be 69.25 minutes, compared with a range of 0.5 to 29.7 minutes for chicken blood found by other investigators, and 5 - 10 minutes considered normal for human blood.

The mean clotting time of plasma from 100 chickens, 10 weeks of age, as determined with chicken brain thromboplastin, was found to be 11.4 seconds, compared with 10 - 300 seconds found by other workers; the mean prothrombin content was 136.6% of human prothrombin levels, as determined by the one-stage prothrombin time technique, but only 53% by the adsorption and elution technique used; and fibrinogen determinations revealed a mean of 345.9 mg./100 ml. of plasma compared with 250 - 400 for humans.

Natural tissue thromboplastin was found to be very active in the chicken. Thromboplastin made from chicken brain was also active but one made from chicken jugular vein was inactive. Although chicken plasma thromboplastin



formation was slow as indicated by prolonged clotting times, the blood cells had some thromboplastic activity.

The coagulation materials prepared from chickens was found to have a great degree of species specificity. Chicken plasma did not clot as readily with coagulation materials made from other species. However, clot retraction was observed in avian blood, although occurring slowly and less completely than in human blood.

One or more natural anticoagulants in avian blood were evident, having the characteristics of antithrombic activity, sensitivity to variations in pH and ionic strength, ability to be adsorbed on large quantities of tricalcium phosphate, and precipitation of one anticoagulant by heating, but retention of another anticoagulant portion even after boiling. The thrombin clotting time of chicken plasma was also found to be sensitive to pH changes, being greatly prolonged in the alkaline range above pH 7.7. This was thought to be due to the anticoagulant content.



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FOREWORD

Mammalian blood coagulation has been the subject of intensive research during the past quarter century stimulated by the discovery of the one-stage prothrombin time by Quick (49). However, avian blood coagulation had not been extensively studied since the work of Delezenne (23) in 1897, until 1958 and 1959 with publications by Wartelle (72) and Didisheim, Hattori and Lewis (25). Other investigators had included observations on some individual phase of avian blood coagulation, but these were incidental to their major work (1, 2, 3, 5, 7, 9, 13, 14, 15, 17, 18, 19, 20, 22, 26, 27, 29, 30, 31, 32, 33, 35, 36, 37, 38, 39, 40, 41, 50, 51, 52, 54, 55, 59, 60, 62, 68.)

Avian blood coagulation was used, however, as a tool to investigate a hemorrhagic diathesis in chicks as observed by McFarlane, Graham and Richardson (45) and Almquist and Stokstad (1, 2). This investigation led to the discovery and assay of Vitamin K by Dam and Schonheyder (54, 55).

Avian coagulation was used as a measure of results in other diverse experiments by: Johnson and Conner (42), studying lymphomatosis in chickens; Warnock et al (70), to correlate exercise with atherosclerosis in cholesterol fed chickens; Barnett et al (9, 10) in studies on B.A.P.N. (beta amino propionitrile); Gray et al (35) and Cover et al (19), while investigating hemorrhagic syndromes in chickens, and Humble and Glover (40), studying avian heparinization.



Since avian blood coagulation may be useful in future work, it was felt that basic information on the subject would avoid many errors that appear to have arisen in previous investigations. To do this, a methodical analysis of blood clotting factors in the chicken was undertaken by application of test procedures that are now routine in the diagnosis and investigation of mammalian coagulation disorders.

The endeavor to cover all of the factors in avian blood coagulation was hampered in the early stages of this investigation by the finding that chicken blood does not behave exactly as does mammalian blood, thus necessitating modification of almost every technique applied. Some facets of avian blood coagulation are presented in this thesis, while a multiplicity of avenues of investigation revealed themselves during the course of the study. It is conceivable that workers could spend their lifetime exploring such avenues as have many in mammalian blood coagulation research.



## INTRODUCTION

Compared with the vast amount of information available on the blood coagulation of man and other mammalian species, comparatively little is known about blood coagulation in the avian species. Near the beginning of the century some work was done in Europe on bird blood, as indicated by references to Delezenne, 1897 (23), and the remarks of Howell, 1909 (37). The pioneering work on tissue culture techniques by Carel and Ebeling (15, 16, 17, 18) stimulated some work on coagulation of chicken plasma, as this was used in their tissue culture experiments. Investigations into thromboplastin led Fischer (29, 30, 31, 32) to investigate techniques in chicken plasma coagulation. This work was basic in the many experiments with chicken blood leading to the discovery, assay and utilization of Vitamin K by many workers, including Dam, Schonheyder, Almquist, Stokstad and Quick (1, 2, 4, 5, 22, 27, 50, 55).

Modern blood coagulation techniques were applied to avian blood by Wartelle in 1957-58 (71, 72), by Soulier, Wartelle and Menach in 1959 (59), and in the same year by Didisheim, Hattori and Lewis (25).

The available work of these previous investigators conveys the following information:

1. Chicken blood clots slowly if a paraffin coated cannula is used and the blood immediately cooled to ice bath temperature. Delezenne (23),

TABLE I

## CHICKEN BLOOD RECORDED CLOTTING TIMES

CLOTTING TIME	TECHNIQUE USED	AUTHOR
Slow	Paraffin coated cannula into ice bath temperature	Delezenne, 1897 (23), Howell, 1909 (37), 1918 (38), 1940 (39), Seegers, 1951 (57).
4.5 minutes	Temperature - 25° C.	Amendt, 1923 (6).
6.3 minutes	Lee-White method-syringe used on wing vein	Johnson & Conner, 1933 (42).
2-4 minutes	Puncture wing vein	Almquist & Klose, 1939 (5).
6 minutes	Cut wing vein-tubes in water bath	Cravens, Randle, Elvehjm, Halpin, 1941 (20).
1-4 minutes	Capillary tube method	Humble & Glover, 1950 (40).
29.7 minutes	Glass tube at room temperature	Stamler & Warner, 1951 (60).
0.5-12.35 min.	Capillary tube method	Jacobs, Elam, Quisenberry, Couch, 1953 (41).
1.5 minutes	Not given	Bornstein & Samberg, 1954 (13)
15 minutes	Fast puncture wing vein-capillary tube method	Cover, Mellen & Gill, 1955 (19).
16 minutes	Lee-White method in glass and silicone	Soulier, Wartelle, Menache, 1959, (59).
9-10 minutes	Lee-White method in glass and silicone at 37° C.	Didisheim, Hattori and Lewis, 1959, (25).



TABLE II

## CHICKEN PLASMA

## CLOTTING TIMES RECORDED WITH VARIOUS CLOTTING AGENTS

CLOTTING TIME	AGENT USED	AUTHOR	COMMENTS
50 seconds	chicken embryo extract	Ebeling, 1919 (26)	
15 minutes	embryo heart, embryo extracts	Carel & Ebeling, 1921 (15,16) 1922 (17,18)	
Variable	extract chicken embryo muscle & lung at 40° C.	Fischer, 1930 (29), 1935 (32)	Great variations in clotting times.
180 seconds	varied concentrations of watery extract chicken lung at 40° C.	Schonheyder, 1936 (55)	Range - 120-600 seconds
180 seconds	varied concentrations of chicken muscle thromboplastin at 39° C.	Dam & Glavind, 1938 (21)	Stock thromboplastin frozen for consistent results.
10-11 sec.	chicken brain thromboplastin	Quick, 1937 (50)	Variations in brain thromboplastin activity.
50 sec. (49.3+3.0)	chicken muscle extract	Almquist & Klose, 1939 (5)	Thromboplastin in whole blood 25-30 seconds.
20-25 sec.	chicken muscle extract	Cravens, Randle & Elvehjem, 1941 (20)	Prothrombin times give inconclusive results.
20/30 tests >300 sec.	Soluplastin (Schieffelin)	Cover, Mellen & Gill, 1955 (19)	Only 10 of 30 chick plasmas clotted >300 sec.

Howell (37, 38, 39) and Seegers (57).

2. Centrifugation of goose blood to remove the thrombocytes made it incoagulable. Delezenne (23).

3. Recorded clotting times of whole chicken blood were extremely variable, ranging from 0.5 minutes to 29.7 minutes, as indicated by several authors in Table I.

4. The inconsistency in coagulation of chicken plasma by various clotting agents was noted by several investigators and is evidenced by the range of plasma clotting time from 11 seconds to over 300 seconds noted in Table II.

5. The prothrombin content of chicken blood appeared to be greatly influenced by the bird's intake of Vitamin K, (Dam, Schonheyder, Almquist, Stokstad and Quick (1, 2, 4, 5, 22, 27, 50, 55)), and is depressed by sulphonamide medication (Asplin and Boyland (7)), even though Vitamin K was found to be synthesized in the intestinal tracts of chickens. (Almquist and Stokstad (3), Almquist and Pendler (4)).

6. Chicken plasma is deficient in antihemophilic factor B (IX) and C. (P.T.A.). (Wartelle (71)).

7. Chicken plasma appears to be deficient in plasma thromboplastin antecedant and Hageman factor

TABLE III  
SUMMARY OF CHICKEN & DUCK BLOOD STUDIES  
Didisheim, Hattori and Lewis (25)

STUDY	CHICKEN	DUCK
Clotting time 37° C. glass (min.)	9	9
silicone (min.)	10	10
Clot retraction	0 - tr	0 - tr
Clot lysis	0	0
Thrombocyte Count ( $\times 10^3/\text{mm}^3$ )	150	324
Platelet prothromboplastin & accelerin	unable to separate thrombocytes	
Prothrombin (%)	4	15
Proconvertin (VII) (%)	0	0
Proaccelerin (V) (%)	5	30
AHF (VIII) (%)	50 #	35 #
P.T.C. (%)	<5	<5
Hageman factor (%)	0	0
Fibrinogen (%)	79	100

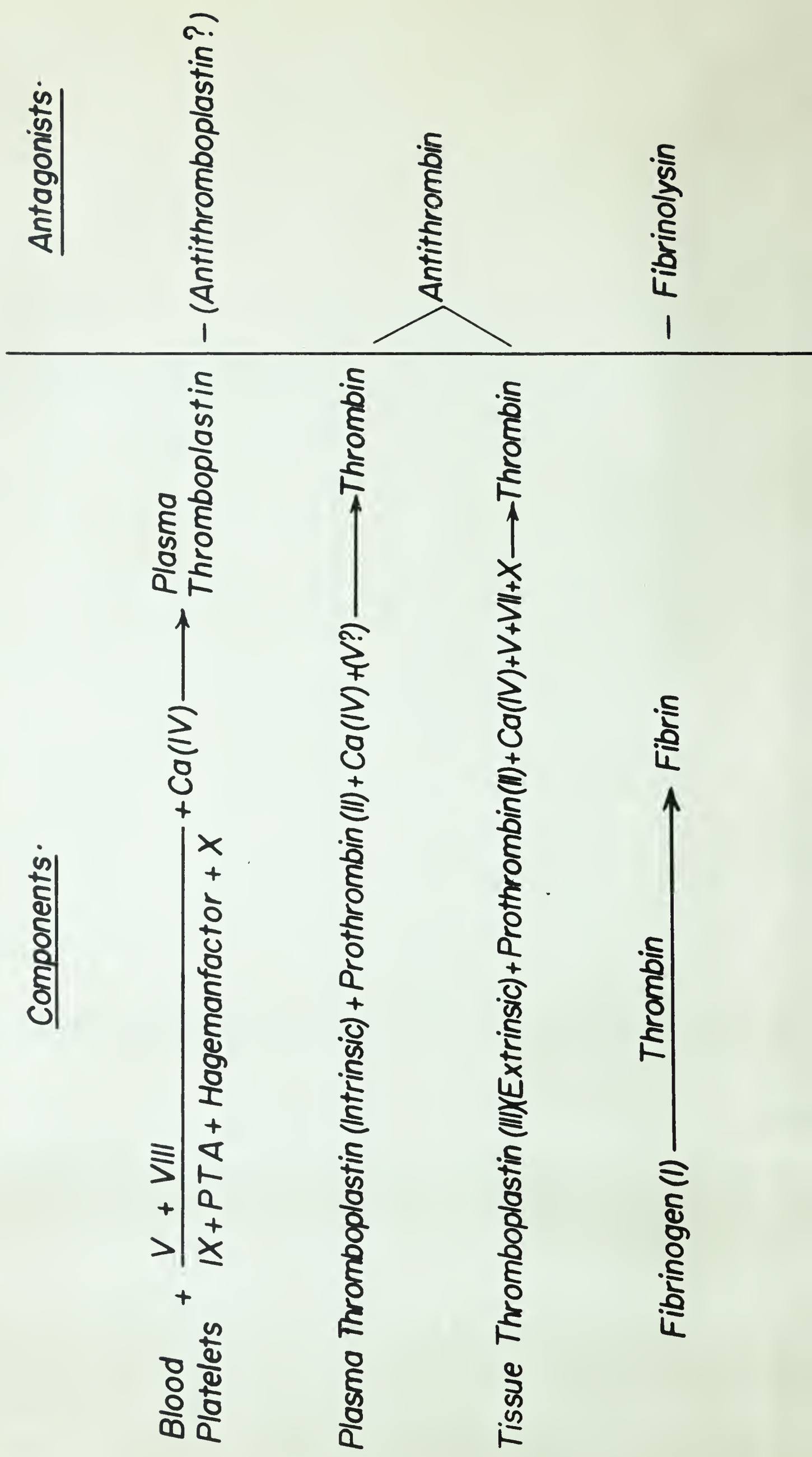
% Percentages compared with man as 100%

# Exact comparisons not possible - restored prothrombin consumption but only shortened recalcification time.



FIGURE I

# Simplified Schema of Blood Coagulation ·





## TABLE aa

## NOMENCLATURE OF BLOOD CLOTTING FACTORS (73)

## FACTOR I:

Fibrinogen

## FACTOR II:

Prothrombin

## FACTOR III:

Thromboplastin (tissue)

## FACTOR IV:

Calcium

## FACTOR V:

Proaccelerin

Labile Factor

Plasma Ac-globulin

Thrombogene

Proprothrombinase

Prothrombokinase

PPCF - Plasmin Prothrombin

Conversion Factor

Component A of Prothrombin

Prothrombin Accelerator

Co-Factor of Thromboplastin

## FACTOR VII:

Proconvertin

SPCA - Serum Prothrombin

Conversion Accelerator

Stabile Factor

Cofactor V

Serozym

Kappa Factor

Prothrombinogen

Co-Thromboplastin

Serum Accelerator

Prothrombin Conversion Factor

Prothrombin Converting Factor

## FACTOR VIII:

Antihemophilic Globulin

Antihemophilic Globulin A

AHF - Antihemophilic Factor

PTF - Plasma Thromboplastic  
Factor

Plasma Thromboplastic Factor A

TPC - Thromboplastic Plasma  
Component

Facteur Antihemophilique A

Thromboplastinogen

Prothrombokinase

Platelet Cofactor I

Plasmokinin

A Thrombokatalysin

## FACTOR IX:

Christmas Factor

PTC - Plasma Thromboplastic  
Component

Antihemophilic Globulin B

Plasma Thromboplastic Factor B

Plasma Factor X

Facteur Antihemophilique B

## FACTOR X:

Stuart - Prower Factor

## Other Factors Described and Under Investigation:

PTA - Plasma Thromboplastic  
Antecedant

Hageman Factor

Glass Factor

(Soulier, Wartelle and Menach (59)).

8. Chicken fibrinogen retards the coagulation of human plasma (Burstein and Guinand (14)).

9. Chicken plasma inhibits clotting of bovine fibrinogen and bovine plasma by bovine thrombin. (Seegers and Smith (58)).

10. Chicken plasma contains a heparin like antithrombin that varies with oestrogen content. (Stamler and Warner (60)).

11. Duck plasma is devoid of Hageman factor and does inhibit the clot promoting effect of glass or barium carbonate upon normal human plasma. (Ratnoff and Rosenblum (53)).

12. Didisheim, Hattori and Lewis (25) used modern coagulation techniques in a comprehensive study of chicken and duck plasma. Their results are summarized in Table III, to which the following observations were added:

- a. slow erythrocyte sedimentation in the chicken and duck.
- b. low hematocrit in the chicken and duck.
- c. thrombocytes are comparable in the chicken and duck to platelets of the mammal although they appear as complete spheroid nucleated cells, three times platelet size.



- d. duck and chicken brain thromboplastin had little activity with most mammalian plasmas.
- e. chicken fibrinogen clotted with only chicken thrombin.
- f. chicken and duck plasma generated no thromboplastin.
- g. platelet poor chicken or duck plasma upon recalcification in glass tubes did not clot in 1 hour. If human platelets were added, clotting slowly occurred.
- h. no inhibiting effect of chicken plasma on normal human plasma found.
- i. the hemostatic mechanism in avian species must be very different from that in the human.

As a basis for avian studies, blood clotting factors as outlined by the simplified schema in Figure I were understood and the numerous synonyms encountered in blood coagulation literature grouped as in Table aa. Many of the findings herein presented are different from those of other investigators.



## MATERIALS AND METHODS

### Donor Birds

Individual fresh blood samples and those for the whole blood clotting tests were obtained from healthy 4 - 8 month old laying hens of three breeds (Light Sussex, White Plymouth Rock and Single Comb White Leghorn), maintained in cage laying batteries on a balanced complete laying mash. For determination of blood volume by exsanguination, birds of various origins and breeds were used. Large quantities of blood were collected at two poultry processing plants from White Rock X New Hampshire broiler type chickens, 10 weeks of age. For accurate estimation of one-stage prothrombin time, prothrombin content by adsorption and elution techniques and fibrinogen determinations, ten 33 ml. blood pools were prepared. Each pool consisted of 3 ml. of blood from each of ten birds and added to 3 ml. of 0.1 M. sodium oxalate solution.

### Equipment

Syringes, B-D needles and vacutainer needles (1 inch, 20 gauge) were silicone coated by sucking G.E. Dri-Film SC-87 organo-silicone through them at least 3 times, thoroughly rinsing with distilled water and drying in air.

Coagulation tests were all conducted with Kimble glass tubes, 13 mm. x 10 cm.

A standard Lipsham water bath was used for all coagulation tests at a temperature of 42° C. This temperature was



chosen as it closely approximated the normal temperature of the chicken, i.e.  $41.47^{\circ}$  C. (61).

Other equipment used included Photovolt Corp. pH meter, Model No. 125B; Beckman pH meter, Model No. H2; Servall refrigerated automatic centrifuge; Coleman Junior Spectrophotometer, Model No. 6A and "Parafilm" paraffin film.

#### Coagulation Materials

Oxalated or citrated blood was prepared by the addition of 1 volume 0.1 M. sodium oxalate solution or 1 volume of 0.2 sodium citrate solution to 9 volumes of blood.

Chicken thromboplastin was prepared by Quick's acetone dehydration method (52). Twenty-one grams of fresh or frozen chicken brains previously cleaned of all blood vessels and tissue were mashed and finely ground in a mortar and pestle with at least four changes of chemically pure acetone. After the acetone had been removed by decantation, vacuum filtration and drying in a bacteriological incubator, 0.2 gram samples of the fine residue were weighed into rubber stoppered tubes, evacuated of air with a vacuum pump, sealed with paraffin and stored at  $-25^{\circ}$  C. Before use 5 ml. of normal saline was added and the mixture incubated in a vacuum bottle at  $50^{\circ}$  C. for 30 minutes.

Chicken thrombin was prepared by a method based on that of Biggs and Macfarlane (12). One hundred ml. lots of



frozen citrated or oxalated pooled chicken plasma were thawed in the water bath, diluted with 900 ml. of distilled water, and adjusted to pH of 5.3 with 50% acetic acid. The precipitate was removed by centrifugation, dissolved in 50 ml. of 0.85 percent saline solution, and then adjusted to a pH of 7.0 by the addition of small quantities of 33 1/3 per cent sodium carbonate solution. The beaker containing this mixture was placed in the water bath at 42° C., 6 ml. of 0.25 M. calcium chloride added, and a glass stirring rod placed in the solution. Fifteen minutes after the addition of the calcium, the rod was rotated in an effort to wrap the clot around the rod to facilitate expressing and removing the fibrin. Clotting occurred at varying intervals from 20 to 40 minutes after the addition of calcium. Once the clot started to form, coagulation was completed rapidly, generally precluding satisfactory wrapping of the clot. In most cases by extreme care and pressure on the jelly-like clot, the majority of the fluid could be expressed and the fibrin clot removed. The remaining fluid containing thrombin was held overnight in the refrigerator or at room temperature for 1½ hours to ensure complete thrombin formation, then was centrifuged to remove sediment and purified by the addition of 1 volume of c.p. acetone to 1 volume of thrombin material. The precipitate was separated by centrifugation and thrombin extracted from it with 25 ml. of 0.85 percent saline solution. This was again centrifuged, the precipitate discarded, and



the supernatant used as thrombin material.

Variations from the original method of Biggs and Macfarlane include: 1. a double dilution with saline prior to recalcification. This was done in an attempt to more satisfactorily remove the fibrin clot. When the original method was used, the clot was solid and transparent, similar to gelatin and no clot could be wrapped from it. Extraction was tried by means of centrifugation and manual expression with glass rods which was only partially satisfactory, as indicated by the small yield. 2. the addition of the calcium chloride to the euglobulin at 42° C. was found to accelerate clot formation. When this procedure was tried at room temperature, clot formation took several hours. 3. the amount of added calcium chloride solution was quadrupled. This was necessary partially due to the added dilution, also that a more firm fibrin aggregate would occur (67), and the better extraction would give a higher thrombin yield.

Although human thrombin prepared according to this method contains approximately 200 N.I.H. units per ml. (12); the chicken material was so weak it had to be used at full strength.

Chicken fibrinogen was prepared by ammonium sulphate fractionation from citrated chicken plasma. To 200 ml. of plasma, 66 ml. of saturated  $\text{NH}_2\text{SO}_4$  was added, to give a final saturation of 25%. The precipitate was removed by



centrifugation, diluted in normal saline and dialized in a dialyzing membrane tube against normal saline, until the dialysate remained clear upon addition of barium sulphate. The resultant material had 0.2 M. sodium citrate added in a ratio of 1:9, was labelled as "chicken fibrinogen" and stored at  $-25^{\circ}$  C. Tyrosine assay revealed 5,952 milligrams of fibrinogen per 100 ml.

Plasma "Fraction II" was precipitated from the plasma supernatant following the removal of the chicken fibrinogen by the addition of an added 33 ml. of saturated  $\text{NH}_2\text{SO}_4$  per 100 ml. of plasma supernatant, calculated to give a final saturation of 37 percent ammonium sulphate. The precipitate thus obtained was removed by centrifugation and dialized against saline and saline-sodium citrate in conjunction with the chicken fibrinogen. Tyrosine assay of this fraction revealed 2,046 milligrams of protein read as fibrinogen per 100 ml.

#### Tests and Determinations

Quantitative estimation of fibrinogen was based on the method of Quick (52) with some modifications. One ml. of oxalated or citrated chicken plasma was diluted with 50 ml. of distilled water in a beaker placed in a water bath at  $42^{\circ}$  C. Two ml. of 0.4 M. calcium chloride was added and a glass stirring rod placed in the beaker. A clot was visible within 45 minutes, which was wrapped around the glass stirring rod, thoroughly washed with distilled water,



removed from the rod, and later assayed for tyrosine content. The modifications from the method of Quick are:

1. Extra dilution of the plasma in 50 ml. of distilled water instead of 25 ml., which was necessary to obtain a fibrin clot that could be wrapped on the glass rod.

2. An increase in the amount of calcium chloride from 2 ml. of 0.1 M. calcium chloride to 2 ml. of 0.4 M. calcium chloride in order to increase the tensile strength of the fibrin clot (67).

3. The test conducted at 42° C. instead of room temperature to speed clot formation.

Determination of prothrombin by the adsorption and elution technique was also based on that of Quick (52).

The tricalcium phosphate from 10 ml. of 0.6 M. solution was packed by centrifugation, the supernatant decanted off and the tube drained. Ten ml. of oxalated fresh chicken plasma was added to the packed tricalcium phosphate, covered with a square of "Parafilm", shaken for at least 5 minutes, then permitted to stand with occasional inversion for an additional 10 minutes. The tricalcium phosphate holding the adsorbed material was separated from the plasma by centrifugation at 5,000 r.p.m., the plasma decanted and the tube dried. Ten ml. of normal saline was added to the packed tricalcium phosphate, covered with "Parafilm" and shaken in order to wash off the remaining plasma. The tricalcium phosphate was



again packed by centrifugation, the saline decanted off and the tube drained and dried with a paper towel. The material adsorbed on the tricalcium phosphate was then removed by elution with 1 ml. of 0.2 M. sodium citrate solution, each 0.1 ml. of eluate now containing prothrombin from 1.0 ml. of plasma. One volume of the eluate was reconstituted by the addition of 9 volumes of fresh adsorbed chicken plasma, a one-stage prothrombin time of the resultant mixture determined, and the prothrombin content estimated by the use of a prothrombin table.

The one-stage prothrombin time of Quick (52) was used, employing chicken thromboplastin and 0.1 or 0.2 M.<sup>sol.</sup>/of calcium chloride.

Thrombin times were determined by the method of Quick (52), utilizing chicken thrombin or bovine thrombin where specified.

The thromboplastic activity of various agents was tested by adding measured amounts to aliquots of euglobulin (Biggs and Macfarlane (12)) at 42° C. prior to recalcification and recording the clotting time.

Determination of the pH of chicken whole blood was conducted on 5 ml. samples taken by the siliconized needle technique from the brachial vein of 4 different breeds of hens, directly into open polyethylene cups. The pH was determined immediately, the meter previously having been



calibrated for an electrode temperature of 40° C. The blood sample was left in contact with the electrodes and read at varying intervals to detect pH change due to air exposure, the temperature calibration at each reading being adjusted accordingly.

Preparation of imidazole buffers of the same ionic strength was accomplished utilizing the method of Mertz and Owen (47), modified so that the ionic strength was equal at each pH.

Tests of chicken plasma fractionated by heating to precipitate fibrinogen and other components were conducted as follows: oxalated chicken plasma from 10-week old birds was divided into 8 ml. lots, heated in thermos bottles for  $\frac{1}{2}$  hour at 45, 50, 55, 60, 65, 70, 75 and 100 degrees C., then centrifuged to remove the precipitate. Two ml. of each heated plasma supernatant was mixed with 0.5 ml. of chicken fibrinogen and 1.25 ml. of pH 6.6 imidazole buffer. Thrombin times were conducted on each heated plasma mixture, utilizing 0.2 ml. of the plasma mixture with 0.1 ml. of chicken thrombin at 42° C.

TABLE IV  
CHICKEN BLOOD COAGULATION TIMES  
COLLECTED USING PLASTIC OR SILICONIZED  
GLASS SYRINGES

NO.	BREED	TIME ( MINUTES)
1	LH	3.0
2	LH	1.5
3	LH	3.0
4	LH	7.5
5	LH	10.0
6	WR	11.5
7	WR	5.5
8	WR	10.0
9	LH	13.5
10	WR	23.25
11	WR	30.0
12	WR	15.5
13	LH	4.5
14	WR	15.0
15	WR	5.5

Range 1.5 - 30 minutes

LH - Single Comb White Leghorn

WR - White Plymouth Rock

COAGULATION TIME OF WHOLE BLOOD

The mean clotting time of 37 laying hens was found to be 69.25 minutes, in contrast to the shorter clotting times found by other investigators (Table I). In a preliminary test of clotting times on 15 chickens, using the Lee-White method (44) with plastic or siliconized glass syringes and siliconized 20-gauge needles, a range of 1.5 to 30 minutes was found at 42° C. (Table IV). It was observed that the negative pressure of the syringe caused a "fluttering" of the fragile vein against the bevel of the needle and, that the greater the degree of "fluttering", the shorter was the clotting time of the blood. It was assumed from this that the trauma caused by the vein striking the needle released tissue thromboplastin from the endothelial lining of the vein which hastened clotting.

To confirm this suspicion, blood from 37 birds was collected, utilizing precautions to minimize the release of tissue thromboplastin by venipuncture with #20 siliconized B-D vacutainer needles only. No syringe was used, the blood being allowed to flow directly into 13 mm. x 10 cm. Kimble glass tubes by very slight digital pressure on the superior portion of the brachial vein. Needle movement within the vein was minimized. Three blood samples of 1 ml. were taken from each of the right and left wings. The first sample taken was not recorded due to the possibility of contamination

TABLE V  
CHICKEN BLOOD COAGULATION TIMES AT 42° C.  
(MINUTES)

Blood collected without syringe using siliconized needle only.

NO.	BREED	LEFT WING		RIGHT WING		Longest time any tube
		Tube II	Tube III	Tube II	Tube III	
1	WR			9	<u>60</u>	60
2	LH	<u>65</u>	45	10	44	65
3	LH	29	41	<u>43</u>	33	43
4	LH	25	<u>29</u>	16	25	29
5	LH	60	<u>62</u>	42	59	62
6	WR	43	43	42	<u>44</u>	44
7	WR	73	73	79	<u>79</u>	79
8	WR	116	<u>120</u>	clot	in needle	120
9	WR	54	<u>54</u>	51	51	54
10	WR	34	<u>34</u>	31	31	34
11	LS	53	<u>53</u>	23	22	53
12	LS	43	<u>43</u>	clot	in needle	43
13	WR	60	<u>60</u>	59	11	60
14	LS	56	<u>56</u>	42	42	56
15	LS	24	<u>35</u>	35	35	35
16	LS	25	<u>25</u>	20	17	25
17	LS	13	<u>13</u>	5	12	13
18	LH	84	84	88	<u>153</u>	153
19	LH	72	120	155	<u>180</u>	180
20	LH	65	<u>65</u>	45	60	65
21	LH	25	<u>60</u>	clot	in needle	60
22	LH	70	70	75	<u>75</u>	75
23	LH	110	140	115	<u>145</u>	145
24	LH	58	58	45	<u>80</u>	80
25	WR	40	45	45	<u>50</u>	50
26	LH	35	<u>35</u>	28	28	35
27	WR	55	<u>85</u>	65	70	85
28	WR	60	60	70	<u>85</u>	85
29	WR	80	90	90	<u>90</u>	90
30	WR	110	<u>110</u>	80	80	110
31	WR	60	60	60	<u>60</u>	60
32	WR	36	36	50	<u>50</u>	50
33	WR	50	105	120	<u>120</u>	120
34	LH	75	<u>75</u>	70	70	75
35	LH	65	<u>65</u>	60	60	65
36	WR	45	<u>65</u>	40	40	65
37	LS	25	<u>40</u>	23	23	40
Arithmetic Mean or Average						69.25

Mean deviation from average -27  
 +5  
 Algebraic sum of deviations 36.6  
 Standard deviation  
 Range - 13 - 180 min.



FIGURE II  
HISTOGRAM OF BLOOD CLOTTING TIMES OF  
37 LAYING HENS SHOWING A RANGE OF  
13 - 180 MINUTES

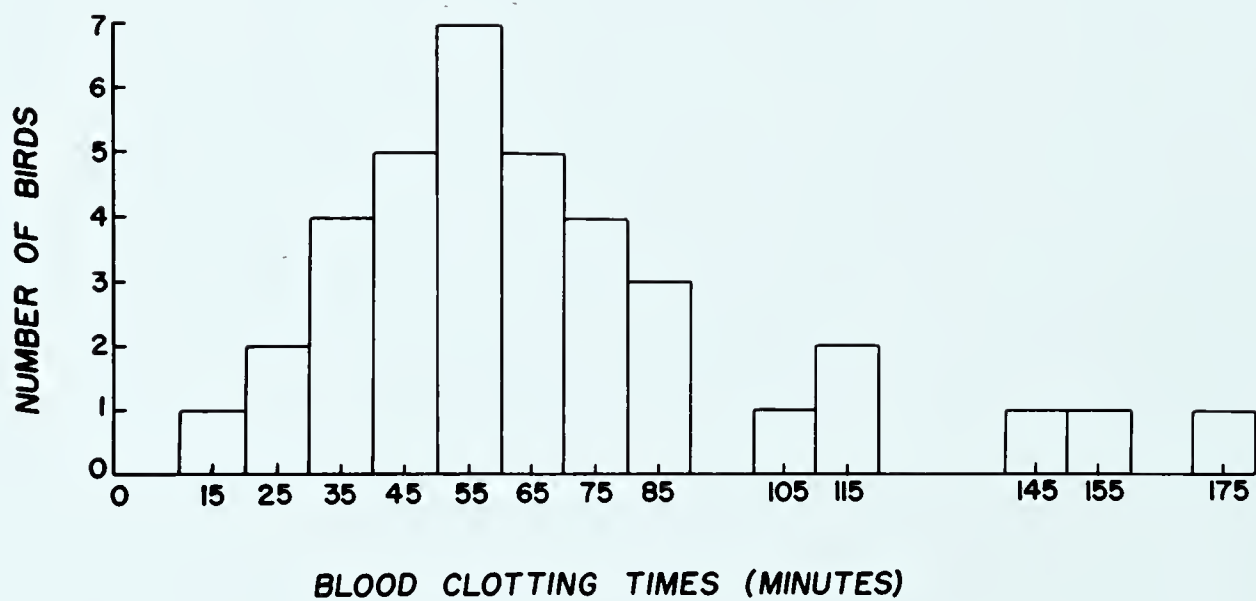




TABLE VI

COAGULATION TIME AT 42° C.

CHICKEN BLOOD WITH AND WITHOUT ADDED THROMBOPLASTIN OR THROMBIN

NUMBER OF TESTS	MATERIAL ADDED	CLOTTING TIME (seconds)	CONTROL LEE-WHITE CLOTTING TIMES (minutes)
21	Chicken Thromboplastin	Mean 13.4 (Range 11 - 17.8)*	70
15	Chicken Thrombin	Mean 30.6 (Range 19 - 50)*	64.5

\* Times varied from wing to wing, possibly indicating tissue thromboplastin activity.

with tissue juices. The clotting times varied a great deal from wing to wing and from tube to tube from the individual wing. Variation was pronounced if any additional trauma was needed to enter the veins satisfactorily, or if needle movement within the vein was excessive, due to struggling by the birds. This is illustrated by birds 8, 12, and 21 in Table V, where difficulty was encountered on venipuncture of the right wing vein resulting in rapid clotting, while prolonged clotting was found from the left wing veins where sharp, clean venipuncture was most easily performed. The tube with the longest clotting time was thought to be the least contaminated with tissue thromboplastin and the closest to the true clotting time for the individual birds. These ranged from 13 to 180 minutes (Figure II) with a mean of 69.25 minutes (S.D. 36.6).

In order to eliminate the possibility of Vitamin K deficiency or other defects in the blood clotting mechanism of the birds tested, native blood was also timed with a stop watch following the addition of 0.1 ml. of chicken thromboplastin and 0.1 ml. of chicken thrombin (full strength). Clotting occurred in seconds after the addition of either material, compared with time of over an hour by the Lee-White method (Table VI). The clotting times with both thrombin and thromboplastin addition varied from the right to left wings from the same bird, possibly indicating contamination with tissue thromboplastin.

TABLE VII

COMPARISON OF CHICKEN CLOTTING TIMES AT 37° C. &amp; 42° C.

37° C.

42° C.

TEST NO.	BREED	LEFT WING		RIGHT WING		LONGEST TIME ANY TUBE (min.)	LEFT WING		RIGHT WING		LONGEST TIME ANY TUBE (min.)	
		Tube II	Tube III	Tube II	Tube III		Tube II	Tube III	Tube II	Tube III		
1	LH	97	<u>117</u>	88	114	117	84	84	88	<u>153</u>	153	
2	LH	180	180	<u>180</u>	177	180	72	120	55	<u>180</u>	180	
3	LH	105	<u>105</u>	60	90	105	65	<u>65</u>	45	60	65	
4	LH	40	<u>40</u>	1.0 clot in needle	1.0 clot in needle	40	25	<u>60</u>	1.0 clot in needle	1.0	60	
5	LH	80	80	85	<u>90</u>	90	70	70	75	<u>75</u>	75	
6	LH	159	<u>215</u>	95	150	215	110	140	115	<u>145</u>	145	
7	LH	58	<u>83</u>	80	80	83	58	58	45	<u>80</u>	80	
AVERAGE LONGEST CLOTTING TIME						118.5						108.3

To assess the difference between water bath temperatures of 42° C. and 37° C., a small series of duplicate blood samples were tested. The results were not consistent (Table VII) but indicated only a slightly longer time at 37° C.

Attempts were made to obtain chicken blood completely free of thromboplastin by means of venous polyethylene catheterization. Three methods of catheter insertion were used - (a) by incision into the exposed vein or through the lumen of a large needle inserted in (b) the jugular vein or (c) the brachial vein. The samples taken by catheter clotted faster (maximum 15 minutes) than samples from the same bird taken by the siliconized needle technique.



TISSUE THROMBOPLASTIN

Evidence of an active natural tissue thromboplastin in the birds tested was based on the following:

1. The repeated observation that trauma to the fragile veins on blood collection with a syringe increases the clotting time in proportion to the amount of "fluttering" or other trauma.
2. Damage to the endothelial lining of the veins by catheterization accelerated the speed of clotting.
3. If blood were collected, taking particular care to avoid contamination with tissue thromboplastin, the coagulation time was over one hour (Table V), whereas, if no precautions were taken to avoid tissue thromboplastin contamination, blood coagulation occurred more rapidly (Table IV).
4. If blood was drawn from the chickens by means of cardiac puncture without the use of an anticoagulant in the syringe, blood clotting was rapid, sometimes occurring within the syringe before the desired quantity had been collected.

Table II lists some chicken tissues that have been used as artificial thromboplastic agents. Fischer (32) placed the thromboplastic ability of various chicken organs in the following order: lung, muscle, heart, kidney, spleen, brain and liver. Quick (50) used dehydrated chicken brain thromboplastin successfully.

TABLE VIII

## COMPARISON OF THROMBOPLASTINS

SINGLE STAGE PROTHROMBIN TIMES OF TWO FRESH CHICKEN PLASMAS  
(SECONDS)

THROMBOPLASTIN	PLASMA	
	BIRD 1	BIRD 2
.075 gm. chicken vein thromboplastin mixed with 1.85 ml. saline	40.8 (4)	238 (2)
0.2 gm. chicken brain thromboplastin mixed with 5 ml. saline	10.2 (6)	12.2 (6)
Saline control	39.0 (2)	163 (4)
0.2 gm. chicken brain thromboplastin mixed with .5 ml. distilled water	12.0 (5)	13.3 (5)
0.15 gm. chicken brain thromboplastin mixed with 5 ml. saline	10.8 (2)	12.1 (5)
0.2 gm. rabbit brain thromboplastin mixed with 5 ml. saline	43.0 (1)	53.7 (3)

( ) Figures in brackets indicate number of tests averaged.

Considering the strong thromboplastic activity of traumatized vein, an attempt was made to make a tissue thromboplastin from chicken veins. Jugular veins were collected from freshly killed chickens by inserting a stainless steel Steinman pin down the lumen of the jugular vein, and by traction separating the vein from the surrounding tissue. The veins were washed and further adhering tissue was removed, while still on the stainless steel pin. They were then stored at  $-25^{\circ}$  C. During preparation of vein thromboplastin from the thawed jugular veins by means of acetone dehydration of Quick (52), it was noted that on decantation of the used acetone, a large amount of fine material was also removed, leaving a resilient "stringy" vein residue which necessitated chopping with scissors. Three grams of thawed jugular vein yielded 0.175 grams of dehydrated vein thromboplastin. This was mixed for use in the same saline ratio as brain thromboplastin. Table VIII compares the efficacy of the chicken vein thromboplastin with chicken brain thromboplastin. No thromboplastic activity appeared to be present. This may indicate that, similar to the findings of Astrup and Bulak (8) with animal arteries, the thromboplastic activity of chicken veins is confined to the endothelium. This could have been removed in the acetone dehydration process.

Quick (52) in the assay of Vitamin K prepared chicken brain thromboplastin by mixing 0.15 grams of dehydrated chicken brain with 5 ml. of saline. This amount was compared



with the 0.2 gram amounts of dehydrated chicken brain that were used routinely in our testing. As shown by Table VIII, very little difference in clotting time was evident.

In one series of prothrombin determinations, it was thought that the thromboplastin may have been mixed for use with distilled water instead of saline. To check the possible effect of such a procedure, chicken thromboplastin prepared with distilled water was compared with that prepared with saline. As indicated by Table VIII, clotting time with the distilled water mixed thromboplastin was delayed up to 1.8 seconds, emphasizing the need for saline in chicken thromboplastin preparation.



PLASMA THROMBOPLASTIN ACTIVITY

The prolonged clotting time of blood from the hens tested showed that little plasma thromboplastin was generated, as compared with mammals, thus raising the question of the role of chicken blood cells in hemostasis. There appears to be some thromboplastic ability present in chicken blood cells, but this is relatively inactive in blood clotting.

The thrombocyte of the chicken is understood to be directly involved in blood coagulation as is the blood platelet in the mammalian system (Sturkie (61); Olson (48)). If this were true, the thrombocyte should be a fundamental component in the formation of plasma thromboplastin (Figure I). The fact that chicken blood clots at all would appear to indicate either contamination with, or the release of tissue thromboplastin, or else the formation of plasma thromboplastin.

In previous literature on avian thrombocytes, Delezenne (23), as quoted by Quick (52), removed all cells including thrombocytes from goose plasma by centrifugation to prevent clotting; Didisheim, Hattori and Lewis (25) found the separation of chicken and duck thrombocytes from other blood cells was difficult, while Denington and Lucas (24), indicated that half the buffy coat in chickens was composed of thrombocytes.

In an effort to assess the thromboplastic activity of chicken blood cells or mixtures of cells containing thrombocytes, a test was devised on the theory that the

TABLE IX  
THROMBOPLASTIC ACTIVITY TEST <sup>x</sup>

MATERIAL ADDED	MICROSCOPIC EXAMINATION	CLOTTING TIME FOLLOWING RE-CALCIFICATION (SECONDS)
Saline		805
Chicken thromboplastin		56
Human platelet factor*		307
Chicken serum		1200
Chicken blood cells separated by settling in saline 1	Equal quantities w.b.c. & r.b.c.	315
" " " " " 2	Approx. 1/3 w.b.c. 2/3 r.b.c.	283
" " " " " 3	Mostly r.b.c. but many w.b.c.	340
" " " " " 4	Mostly w.b.c.-many stripped nuclei	480
" " " " " 5	Mostly w.b.c.-few r.b.c. many stripped nuclei	450
" " " " " 6	Mostly r.b.c.	360

<sup>x</sup> 0.1 ml. of various materials added at 42° C. to 0.5 ml. chicken euglobulin prior to recalcification with 0.1 ml. of 0.25 M. CaCl<sub>2</sub>)

\* From Warner-Chilcott T.G.T.R. test kit.

addition of a material with any thromboplastic activity should shorten the clotting time of euglobulin upon recalcification in proportion to that activity. Separation of cells by centrifugation at speeds of 1,000 r.p.m. and higher was found to be unsatisfactory, as the addition of centrifuged plasma to euglobulin exhibited little thromboplastic activity. Cell separation was also attempted by high speed centrifugation, substitution of the plasma with normal saline, thorough mixing of the blood cells and saline, and then separation by settling at 4° C. A second saline separation technique was tried by suspending the buffy coat from a centrifuged sample of chicken plasma in saline which was allowed to settle at 4° C. Table IX shows the clotting time of recalcified euglobulin after the addition of chicken blood cells separated by the saline settling technique, compared with the addition of chicken thromboplastin, human platelet factor or chicken serum. The addition of any combination of the chicken blood cells used, accelerated clotting of the euglobulin when compared with the saline control, and was equal in thromboplastic activity to the human platelet factor. All samples of chicken blood cells contained some thrombocytes when examined microscopically. As thrombocytes are thought to be fragile and agglutinate quickly (Frederickson, Chute and O'Meara (33)), the many stripped nuclei found may have been damaged thrombocytes which had taken part in the generation of plasma thromboplastin, although the erythrocytes may have contributed some

TABLE X  
NATIVE CHICKEN BLOOD AND PLASMA <sup>x</sup>  
(Hours)

BIRD NUMBER	WHOLE BLOOD	NATIVE PLASMA LEFT ON TOP OF BLOOD CELLS	NATIVE PLASMA SEPARATED FROM CELLS
1	-	7.5	> 23
2	-	> 7.5	> 23
3	1.25	3.4	> 60
4	0.75	2.4	9
5	0.2	1.4	1.4
6	> 1.3	3.9	> 12.25
7	26.3	44	92
8*	.33	0.5	0.5
9	-	8	120

<sup>x</sup> Comparative clotting time at room temperature in glass tubes.

\* Clot in needle - tissue thromboplastin contamination.

thromboplastic activity.

Table X gives a comparison of clotting times of native chicken whole blood, native chicken plasma left on top of precipitated blood cells, and native plasma removed from the blood cells. In every recorded instance, the whole blood clotted more rapidly than either the native plasma with or without cells. In nearly every instance, also the native plasma left in contact with the precipitated cells clotted more rapidly than the native plasma removed from the cells. This would indicate that the presence of blood cells accelerated the clotting, or that some factor was prolonging the clotting time of the plasma once it was separated from the cellular blood constituents. It may be postulated, as did Delezenne (23), that the removal of the thrombocytes was the prime factor in preventing clotting, although, in one instance, native plasma was diluted approximately five times with distilled water, calcium added at 42° C. and rapid clotting occurred as though some anticlotting factor had been diluted.



THROMBIN PRODUCTION AND TESTING

After many difficulties, a weak chicken thrombin was produced that had to be used full strength instead of allowing dilution of 1:20 as with human thrombin prepared by the same method. Chicken plasma thrombin times varied with pH, but if a pH 6.2 imidazole buffer was used, a mean of 12.14 seconds was obtained on plasma from one hundred 10-week old chickens.

Early in the experiments with chicken plasma, it was found that coagulation with commercial bovine thrombin was unsatisfactory, giving greatly extended clotting times. To overcome any possible species specificity, preparation of chicken thrombin was undertaken utilizing the method of Biggs and Macfarlane (12). Thrombin production from chicken plasma was fraught with many obstacles, the major of these being a greatly prolonged clotting time of the euglobulin following recalcification, and a solid transparent "jelly-like" consistency to the clot once formed, which rendered removal of the fibrin by wrapping on a glass rod impossible, due to crumbling of the material. As previously indicated, the speed of clotting was increased by incubating the material at 42° C. Doubling the dilution of the euglobulin prior to recalcification resulted in a clot that could be wrapped, but was too fragile for complete expression of the fluid material. An increase in strength of the fibrin strands rendering a more satisfactorily "wrappible" clot was accomplished by using four times the



amount of calcium suggested in the original method. Even then, once clot formation was initiated in the euglobulin, it moved to conclusion very rapidly, interfering with fibrin clot wrapping that is accomplished easily in the production of human thrombin.

Where human thrombin prepared by this method was assumed to contain 200 N.I.H. units per ml. (12), the chicken thrombin was considerably weaker. Actual assay of the thrombin by the methods of Quick (52) or Seegers (56) appeared to be precluded by the sensitivity of the chicken thrombin time to changes in pH, and by species specificity of the coagulation materials. One preliminary test comparing chicken thrombin, full strength, and bovine thrombin at the dilution of 1 N.I.H. unit per 0.1 ml., revealed a clotting time of rabbit plasma with bovine thrombin of 5 seconds, and with chicken thrombin of 10 seconds. Over 150 seconds were needed to clot chicken plasma with bovine thrombin, but it clotted in 20 seconds with the chicken thrombin. This experiment indicated the advisability of using the chicken thrombin at full strength for future tests. This was further confirmed by thrombin tests on chicken fibrinogen (Table XIX) and pools of fresh chicken plasma (Table XIV).

In an effort to improve the yield of thrombin from the chicken plasma and in the belief that the presence of tissue thromboplastin or the formation of plasma thromboplastin may be factors in the amount of thrombin formed,

TABLE XI

## CHICKEN THROMBIN

VARIATION IN STRENGTH DUE TO THROMBOPLASTIN ADDITION

	WITH LEUCOCYTES		WITHOUT LEUCOCYTES	
	1 plain	2 thromboplastin added *	3 plain	4 thromboplastin added *
Euglobulin clotting time following recalcification (minutes)	22.5	4.5	34.5	4.0
Chicken fibrinogen clotting <sup>+</sup> time with thrombin resulting from above (seconds)	8.0	7.4	10.8	7.3

\* 0.2 ml. of chicken thromboplastin to 25 ml. euglobulin.

+ 0.2 ml. undiluted chicken fibrinogen to 0.1 ml. undiluted chicken thrombin

aliquots of oxalated chicken plasma were prepared as follows:

1. Centrifuged at 2,000 r.p.m. in order to contain some cellular elements,
2. Centrifuged at 6,000 r.p.m. to remove all cells.

These were prepared by the method of Biggs and Macfarlane (12) into euglobulin. Each euglobulin was divided into 25 ml. aliquots. To one aliquot from each euglobulin, 0.2 ml. of chicken thromboplastin was added prior to recalcification, at room temperature of 21° C. Table XI shows the shortened clotting times found when thromboplastin was added. The slightly shortened clotting time of 22.5 minutes noted with the euglobulin containing leucocytes, compared with 34.5 minutes for the euglobulin prepared without leucocytes, possibly could indicate some thromboplastic activity in the leucocyte containing plasma. The oxalated plasma used for the preparation of the euglobulin was obtained at slaughter and no doubt contained large quantities of tissue juice contamination. The solid clots formed from euglobulins 2, 3 and 4 (Table XI) rendered extraction of the thrombin difficult, the yield being quite small, despite repeated centrifugation and squeezing of the clot.

The activity of the thrombin formed was tested on a chicken fibrinogen (Table XI) which revealed thrombin activity closely following the speed of initial euglobulin clot formation and presumably thromboplastic activity.



Another attempt at a more satisfactory recovery of thrombin consisted of clotting a similar euglobulin, but instead of removing the fibrin clot, the whole clotted euglobulin was left overnight in the water bath, resulting in complete lysis. Assuming that the thrombin remained after clot lysis, extraction of the thrombin was attempted by the addition of one volume of acetone to one volume of the lysed euglobulin material. The precipitate was packed by centrifugation, dissolved in 0.85% saline to bring it back to original volume, again centrifuged and the supernatant was used as thrombin material for testing. Tests with buffered chicken plasma revealed a clotting time of 10.1 seconds for normally prepared thrombin, whereas the lysed clot thrombin failed to coagulate the chicken plasma after 1,200 seconds. Further work, which will be reported later, indicated the presence of an anticoagulant in the lysed clot thrombin material. However, even with the removal of the anticoagulant by heating at 58° C. for 15 minutes, no thromboplastic activity was evident.



FIBRINOGEN CONTENT

Modification of existing techniques employed for quantitative fibrinogen determination on 100 plasmas from 10-week old chickens, revealed a mean content of 354.9 milligrams per 100 ml. of plasma. This is within the upper range of human fibrinogen contents of 250 - 400 (Quick (52)), and higher than that found by Didisheim, Hattori and Lewis (Table III).

When the quantitative estimation of fibrinogen, based on the method of Quick (52) was used, some difficulties were encountered revolving around the formation and extraction of a satisfactory fibrin clot from the chicken plasma. As with the thrombin production, extra dilution of the plasma, increasing the quantity of calcium and conducting the clotting portion of the procedure at 42° C. instead of at room temperature; gave results that could be considered more satisfactory. At room temperature, clotting was greatly prolonged. If extra dilution and extra calcium was not employed, the clot was transparent and "jelly-like" with the fibrin impossible to harvest. In our first experiments, when this occurred we attempted to harvest the fibrin for tyrosine assay by centrifugation. This technique gave widely divergent results with duplicate samples and was considered unsatisfactory. If the plasma was diluted with saline, in many instances no clot occurred, even though a slight precipitate may have been deposited on the bottom of the beaker. Varying amounts of

TABLE XII  
FIBRINOGEN CONTENT CHICKEN PLASMA\*  
(mg./100 ml. plasma)

POOL# NO.	NO. BIRDS PER POOL	DISTILLED WATER DILUTION	SALINE DILUTION (0.1 ml. thromboplastin added)%
1	10	333	228
2	10	374	286
3	10	358	292
4	10	411	286
5	10	315	142
6	10	294	244
7	10	363	260
8	10	401	260
9	10	363	196
10	10	337	244
100 mean		354.9	243.8

# Aliquots of blood from ten birds per pool

\* From broilers 10 weeks of age

% Tyrosine content of 0.1 ml. thromboplastin deducted from total plasma + thromboplastin tyrosine reading. This is felt to be too great, as it presupposes that all thromboplastin is incorporated with clot - such is not the case.

calcium were tried in order to get a strong "wrappible" clot. It was found that by using four times the amount of calcium indicated in the original method, a fairly speedy, "wrappible" clot could be obtained. The addition of chicken thrombin, chicken thromboplastin and chicken serum was attempted in an effort to improve fibrin yield. Some improvement was found, but the tyrosine content of the added materials interfered with the final reading of the test. Even when these were determined separately and subtracted from the original, the results were not considered to be as satisfactory as when no additional agent was added. The removal of the calcium oxalate by centrifugation prior to the quantitative estimation of the fibrinogen content made very little difference when the results of this procedure were compared with results determined with the small amount of calcium oxalate still present.

Quantitative fibrinogen determinations to compare dilution of plasma in distilled water or saline were made on 10 pools of plasma prepared from 10-week old broiler chickens, each pool containing an aliquot of blood from each of 10 birds. Table XII indicates the mean fibrinogen content was 354.9 milligrams per hundred ml. of plasma by distilled water dilution.

The plasma in distilled water clotted in 45 minutes after recalcification; plasma in saline did not clot when left in the water bath at 42° C. overnight, but then clotted



in 15 minutes when 0.1 ml. of chicken thromboplastin was added. Tyrosine determination on the resultant clot from saline was adjusted by deducting the tyrosine content of 0.1 ml. of thromboplastin. The results given in Table XII indicate a fibrinogen content by the saline dilution method of 243.8 ml. per 100 ml. plasma. This figure is believed to be too low because the tyrosine content of the whole 0.1 ml. of thromboplastin was deducted. However, all of the thromboplastin added was not incorporated in the clot, as a great deal was left in the saline diluent.

Determinations were attempted on single plasma samples from the same group to find a fibrinogen range for this age. One ml. of plasma from the 10 single specimens were diluted, as were the pools, in 50 ml. of distilled water or in 50 ml. of saline. At variance with the results obtained with pools of plasma, some of the single specimens did not clot, even when diluted in distilled water, leaving only a fine precipitate at the bottom of the beaker. If thrombin or thromboplastin were added, clots occurred in the majority. It was felt that the variability in clot formation in the single specimens precluded accurate range determinations and may have been due to variations in the amount of tissue thromboplastin contamination at the time of blood collection.

TABLE XIII  
PROTHROMBIN ESTIMATIONS OF FRESH OXALATED  
PLASMA (QUICK (50, 52))

CLOTTING TIME (SECONDS)	PROTHROMBIN CONTENT IN PERCENT OF NORMAL		
	CHICKEN (50) *	DOG (52)	MAN (52)
6		100	
7		50	
8		40	
9		25	
10 - 11	100%	20	
12		10	100
13	60	7	70
14	55	6	60
15		5	50
16	45		
17	40	4	40
18	37	3.5	35
19 19.5	35	3	30
20	32		
22	27	2.5	25
23	25		
24	22		
25	20	2.0	20
28	19		
30	18		
32	17		
37	14		
40	12	1.0	10
46	10		
50	10		
55 - 65		0.5	5

\* Quick had difficulty at this time obtaining uniform results from chicken thromboplastin and could not establish a prothrombin curve.



TABLE XIV

PROTHROMBIN AND THROMBIN TIMES  
ON CHICKEN PLASMA\* POOLS OR SINGLE SAMPLES<sup>x</sup>

NO.		NO. BIRDS PER POOL	ONE-STAGE PROTHROMBIN TIME# (SECONDS)	THROMBIN TIMES£ (SECONDS)
POOLS OF PLASMA	( 1	10	10.6	12.4
	( 2	10	11.6	12.6
	( 3	10	10.8	12.8
	( 4	10	11.0	10.2
	( 5	10	10.8	10.7
	( 6	10	10.3	11.7
	( 7	10	11.7	11.7
	( 8	10	11.0	12.8
	( 9	10	14.5	13.0
	( 10	10	11.7	13.5
		100	Mean 11.4 (Range 10.3-14.5)	12.14 (10.2-13.5)
PLASMA FROM SINGLE BIRDS	( 1	1	11.2	11.6 <sup>+</sup>
	( 2	1	10.9	12.0
	( 3	1	11.6	10.0
	( 4	1	11.6	12.0
	( 5	1	13.8	14.0
	( 6	1	12.6	16.0
	( 7	1	11.3	17.0
	( 8	1	11.6	15.0
	( 9	1	13.6	14.6
	( 10	1	12.0	11.6
		10	Mean 12.0 (Range 10.9-13.8)	13.38 (10.0-17.0)

\* From 10-week old chickens - stored in ice bath.

<sup>x</sup> All figures are the mean of 3 readings except thrombin times on single plasmas.

<sup>+</sup> Result of one reading 10 hours after collection.

<sup>#</sup> Chicken thrombin and thromboplastin used.

<sup>£</sup> 0.1 ml. of pH 6.2 imidazole buffer added to plasma.

PROTHROMBIN DETERMINATIONS

The mean prothrombin content of plasma from 100 chickens, 10 weeks of age, when determined by three methods and compared with that of man, was: 136.6% by one-stage prothrombin time, 53% by reconstitution of adsorbed eluate with fresh chicken adsorbed plasma and 6.1% when the same eluate was reconstituted with rabbit adsorbed plasma. Only the latter figure is comparable to the findings of Didisheim, Hattori and Lewis (Table III).

Quick (49) utilized the one-stage prothrombin time for the study of a hemorrhagic disease of chicks (50), obtaining an apparently normal clotting time of fresh oxalated plasma with chicken brain thromboplastin of 10 to 11 seconds. Times longer than this were scaled in a percentage of prothrombin content as indicated in Table XIII.

In order to ascertain prothrombin content of oxalated plasma from 10-week old broiler chickens, 10 pools of blood were collected, each containing an aliquot from 10 birds. Ten samples from single birds were also obtained. One-stage prothrombin time and also thrombin time determinations were conducted on these plasma. As shown by Table XIV, the mean prothrombin time of the 10 pools representing 100 young chickens was 11.4 seconds, close to the normal time determined by Quick. The ranges of 10.9 to 13.8 seconds for the single birds and from 10.3 to 14.5 for the pools of plasma, may indicate a true range of prothrombin content, as this is said

TABLE XV

## PROTHROMBIN TIMES OF CHICKEN PLASMA FOLLOWING ADSORPTION

WITH DIFFERENT AMOUNTS OF TRICALCIUM PHOSPHATE \*

EXPERIMENT NO.	UNADSORBED	MOLARITY OF TRICALCIUM PHOSPHATE																
		.005	.0075	.01	.012	.017	.02	.04	.045	.05	.055	.06	.065	.07	.075	.08	.1	.2
1	13.8	15.4	14.5	15.8	15	18												8
2	12.3						16.2	46				>900			>900	>900		
3	14.2							29.5	44.7	46.9	74.5							
4	11.8									81.0	>3600	>3600	>3600	>3600				

\* 1 ml. of tricalcium phosphate solution used to adsorb 1 ml. chicken plasma.

to vary even between summer and winter time feeding (62).

The thrombin times, although still slightly longer than the prothrombin times, are in fairly close agreement. As will be shown later, thrombin times are very sensitive to pH and in preliminary testing on this series where no buffer was used, thrombin times were at least doubled.

Prothrombin determination by the adsorption and elution technique of Quick (52), was made utilizing adsorption on 0.6 M. solution of tricalcium phosphate, compared with 0.005 M. and 0.015 M. solutions used for human and rabbit prothrombin respectively. This comparatively large amount of tricalcium phosphate was used when it was found that the adsorbed plasma, after supposed removal of the prothrombin with 0.005 M. tricalcium phosphate, was still able to clot in 15 seconds after the addition of thromboplastin and calcium, indicating that much still remained. Experiments with varying molarities of tricalcium phosphate for chicken plasma adsorption as outlined in Table XV, indicated that 0.6 M. tricalcium phosphate may be the most satisfactory. As will be presented later, it was found that adsorption with higher molarities of tricalcium phosphate also adsorbed other materials believed to be anticoagulant in nature.

Prothrombin determination by the adsorption and elution technique was performed on the same 10 pools of oxalated chicken plasma as were used for the one-stage prothrombin determination. Aliquots of the eluate from each pool were

TABLE XVI  
PROTHROMBIN ESTIMATIONS OF OXALATED  
CHICKEN PLASMA

ADSORPTION & ELUTION TECHNIQUE						ONE-STAGE TECHNIQUE	
POOL NO.	ADSORBED PLASMA USED FOR RECONSTITUTION	CLOTTING TIME (SECONDS)		ESTIMATION (PERCENT OF HUMAN)		TIME (SECONDS)	ESTIMATION (PERCENT OF HUMAN)
		CHICKEN *	RABBIT +	CHICKEN	RABBIT		
1	Chicken (C)	16.1		45		10.6	169
	Rabbit (R)		65.5		< 5		
2	C	15.1		50		11.6	114
	R		72.0		5		
3	C	14.7		52		10.8	157
	R		37.0		11		
4	C	13.8		62		11.0	143
	R		47.0		5		
5	C	14.6		55		10.8	157
	R		49.0		5		
6	C	14.7		52		10.3	206
	R		46.0		5		
7	C	15.7		46		11.7	110
	R		52.5		5		
8	C	15.6		46		11.0	143
	R		44.0		8		
9	C	16.5		42		14.5	57
	R		56.0		5		
10	C	13.2		80		11.7	110
	R		47.0		7		
ADSORBED C		270					
PLASMA							
CONTROL R		>900					
MEAN				53	6.1		136.6

\* Chicken thromboplastin used on chicken plasma reconstituted eluate  
+ Rabbit thromboplastin used on rabbit plasma reconstituted eluate

reconstituted with fresh adsorbed rabbit plasma or fresh adsorbed chicken plasma. The one-stage prothrombin time being conducted with rabbit thromboplastin on the rabbit plasma reconstituted material and with chicken thromboplastin for the chicken reconstituted material. Table XVI gives the result of this testing together with estimation of prothrombin content, calculated as percent of human prothrombin (Table XIII) for both the adsorption and elution technique and the one-stage prothrombin technique. The great divergence of prothrombin estimation by these methods suggests three comments. Firstly, under the conditions used, the one-stage prothrombin technique is likely to be the most accurate, with 10-week old chickens having a higher prothrombin content than the human, and suggesting that a chicken prothrombin table should be constructed similar to that used by Quick (50) for more correct interpretation. Secondly, compared with the one-stage technique, the adsorption and elution result of 53% is low. The reason may be that some anticoagulant factor is also being adsorbed on the tricalcium phosphate, bringing about prolonged clotting time with subsequent reduction in prothrombin estimation. Thirdly, the adsorption and elution technique, utilizing adsorbed rabbit plasma and rabbit thromboplastin giving a prothrombin estimation of 6.1%, close to the 4% observed by Didisheim, Hattori and Lewis (25). The great difference between the rabbit adsorbed and chicken adsorbed plasma for reconstitution appears to demonstrate evidence of species



specificity, while the possible adsorption of an anticoagulant could also come into play with this determination.

In Quick's original method (52), the adsorbed plasma control should not clot for at least 5 minutes to indicate complete prothrombin adsorption. In the experiment, as indicated by Table XVI, the chicken adsorbed plasma did clot in  $4\frac{1}{2}$  minutes even with the 0.6 M. tricalcium phosphate, indicating good but incomplete prothrombin adsorption.

FIGURE III  
SENSITIVITY OF CHICKEN THROMBIN TIMES  
TO CHANGES IN pH

Determinations of pH made on mixed  
chicken plasma and buffer prior to  
the addition of thrombin.

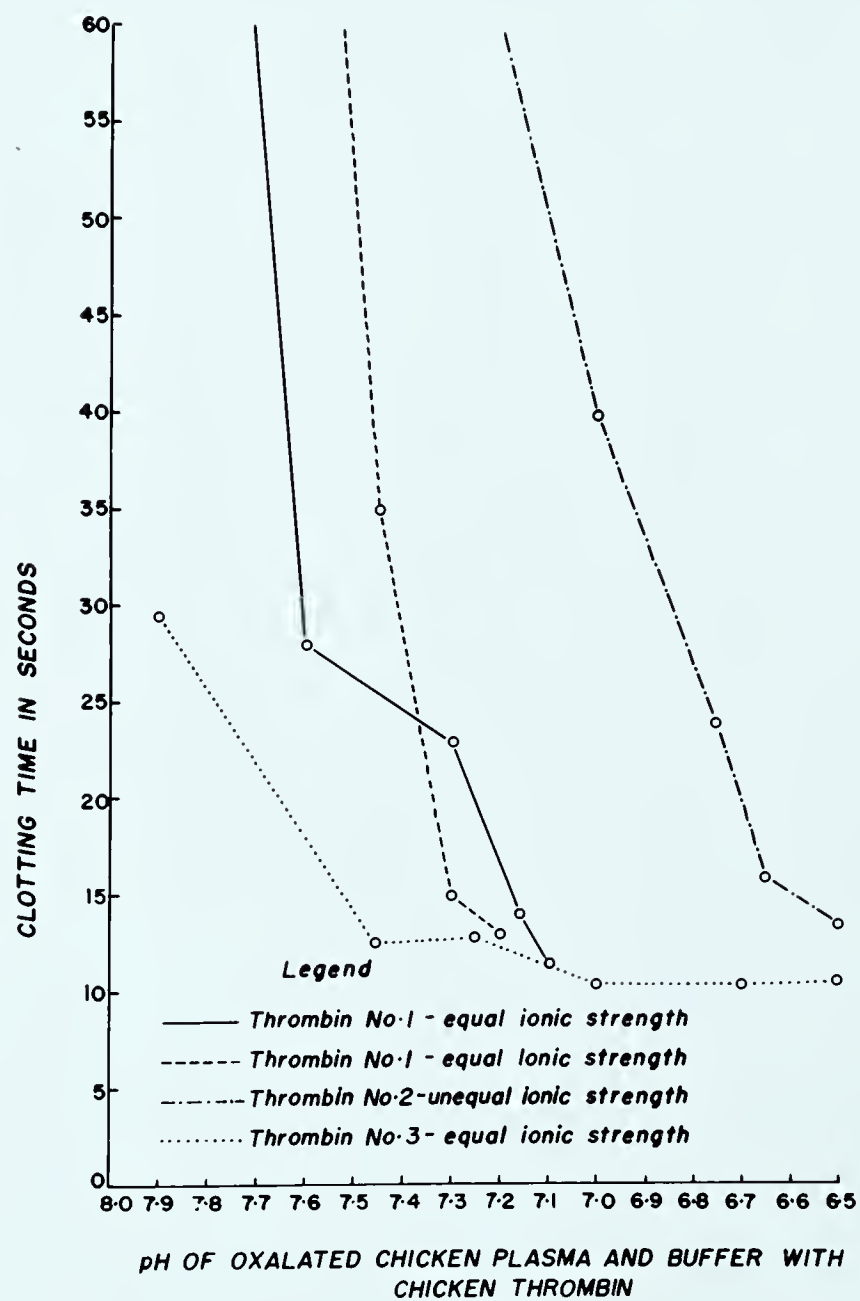
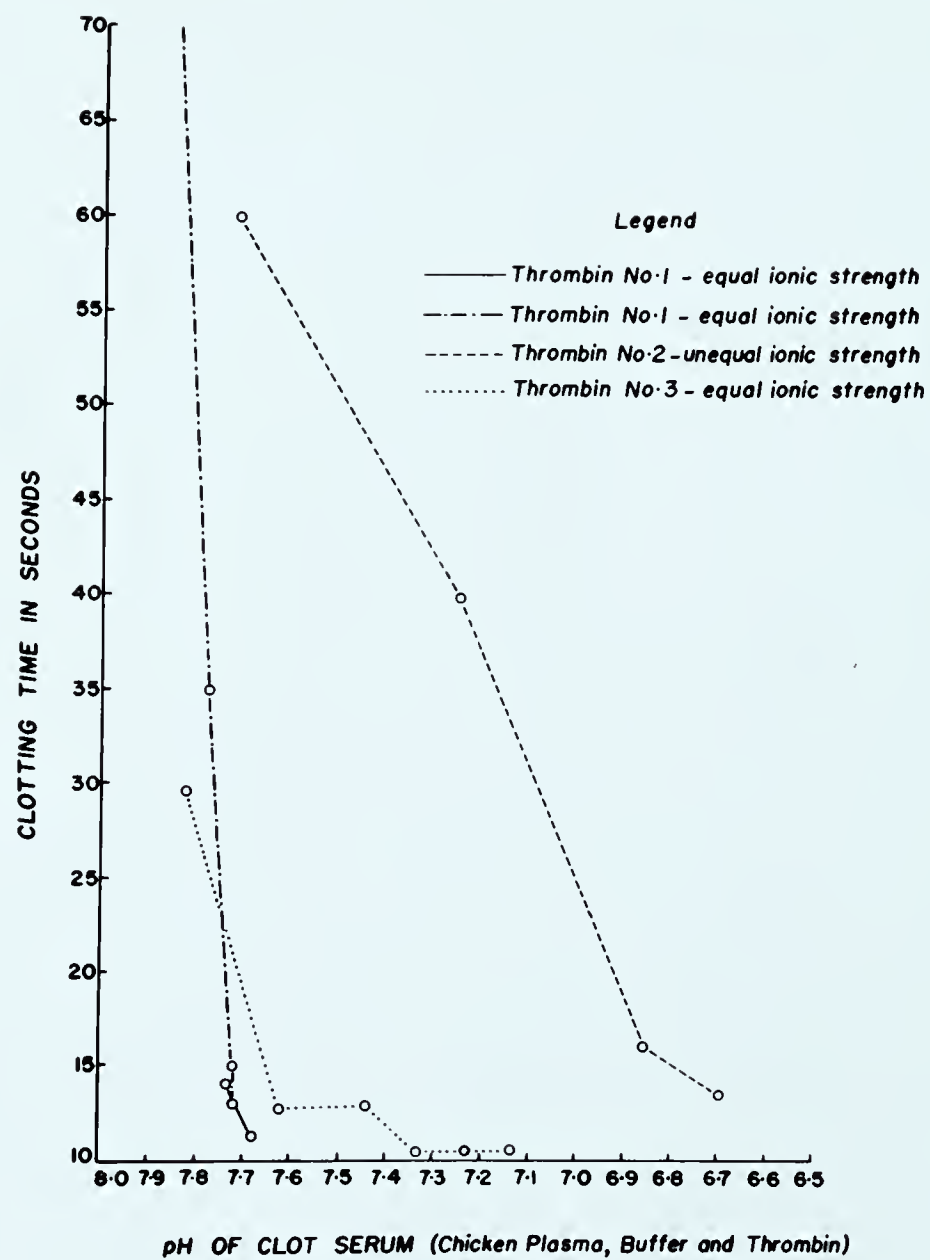




FIGURE IV  
THROMBIN TIMES IN RELATION TO  
FINAL pH OF CLOT SERUM



SENSITIVITY TO pH

Avian blood coagulation was found to be extremely sensitive to variations in pH, especially in the range above 7.7, encountered with oxalated and native plasma.

Early in the course of these studies, it was found that one-stage prothrombin times with rabbit thromboplastin on turkey plasma kept at room temperature, gradually increased from 35 to 72 seconds during  $1\frac{1}{2}$  hours of testing. Bovine thrombin times on the same plasma lengthened from 2 minutes to over 6 minutes after 1 hour exposure at room temperature. Addition of aliquots of 6.4 and 7.3 pH buffers accelerated the bovine clotting time to 7.5 and 10.5 seconds respectively.

To investigate this apparent sensitivity to pH, imidazole buffers were prepared in the pH range of 6.2 to 7.8, utilizing the method based on that of Mertz and Owen (47) with the modification that each buffer was of equal ionic strength. A preliminary test of 0.1 ml. of each of the buffers together with equal quantities of oxalated chicken plasma and bovine thrombin revealed a mean clotting time of 20.5 seconds with the 6.2 imidazole buffer gradually lengthening until clotting time was over 300 seconds with the 7.8 pH buffer or saline. Figures III and IV reveal the extreme pH sensitivity of different oxalated chicken plasmas at varying pH with different chicken thrombins, and illustrating the effect of equal and unequal ionic strength. The pH determinations for Figure III were made on the plasma and buffer mixture prior to

TABLE XVII  
pH FRESH WHOLE CHICKEN BLOOD

TIME AFTER COLLECTION	BIRD NO. & BREED *										MEAN
	1 WR	2 WR	3 WR	4 WR	5 LS	6 LS	7 LS	8 LS	9 RCWL	10 SCWL	
Immediately	7.45	7.56	7.5	7.51	7.6	7.5	7.56	7.6	7.48	7.59	7.535 (R 7.45 - 7.6)
5 minutes	7.5		7.54	7.58	7.68	7.59	7.64	7.66	7.55	7.65	7.599 (R 7.5 - 7.68)
10 minutes	7.53		7.59	7.58	7.69	7.6	7.67	7.68	7.62	7.70	7.628 (R 7.53 - 7.7)
15 minutes	7.56		7.6	7.59	7.69	7.61			7.63		7.613 (R 7.56 - 7.69)
30 minutes	7.56		7.62								7.59 (R 7.56 - 7.62)

\* WR - White Plymouth Rock  
 LS - Light Sussex  
 RCWL - Rose combed White Leghorn  
 SCWL - Single combed White Leghorn

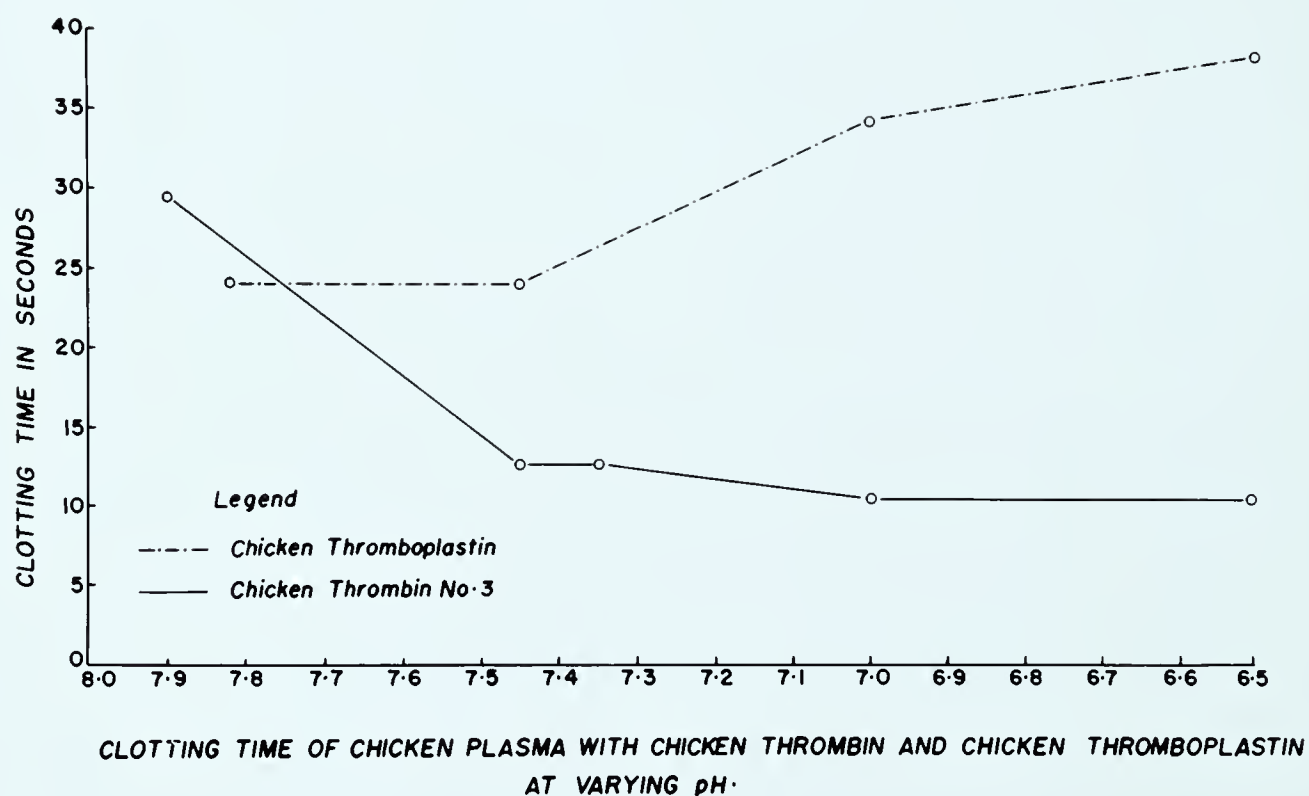
the addition of chicken thrombin. To more accurately assess the final pH of the mixture, determinations were also made on the serum extracted from the final clot. The greatly prolonged clotting times in the alkaline range explains much of the difficulty encountered when conducting thrombin times on unbuffered oxalated chicken plasma. This extreme pH sensitivity is similar to the sensitivity exhibited by anticoagulant fraction of incubated fibrinogen (AFIF) (64). In these experiments the pH of the various materials was adjusted in order to stay within the range of the buffers of equal ionic strength. Due to the alkaline nature of chicken plasma, this sometimes was difficult to do when low pH determinations were required, and so these were obtained by addition of dilute hydrochloric acid, indicated as unequal ionic strength on the graphs.

In the light of these findings, further thrombin time determinations were conducted utilizing a buffer of low pH which reduced the clotting time at least 50%.

Since avian blood coagulation appeared to be very sensitive to changes in pH, the hydrogen ion concentration of fresh chicken blood was determined by an open cup method, as a closed electrode system for pH determination of blood was not readily available. The results in Table XVII, indicate a mean pH of 7.53 on the reading immediately after collection. As the pH rose very slowly, even on exposure to air, there was the possibility that this pH may have been only slightly

FIGURE V  
COMPARISON OF CHICKEN THROMBIN AND THROMBOPLASTIN

Clotting time of chicken plasma  
comparing chicken thrombin and  
chicken thromboplastin in a  
range of pH.



higher than that found in the circulating venous blood. The gradual progression to higher alkalinity was presumably due to loss of  $\text{CO}_2$  content. Seven oxalated chicken plasmas gave a mean pH reading of 7.9 (range 7.8 to 8.0). Two native plasmas gave pH readings of 7.8 and 7.78.

The shorter clotting time noted in native plasma left in contact with precipitated cells may not be due to changes in pH due to buffering by the erythrocytes, as pH determination on one such plasma was 7.8, within the range found for native plasma.

Sensitivity to pH was also exhibited by chicken thrombin and chicken thromboplastin, as indicated in Figure V. Both chicken thrombin and chicken thromboplastin appear to have an optimum action at approximately pH 7.45. Above this pH, thrombin activity appears to decrease, while still being able to function well at pH as low as 6.5. Thromboplastin, on the other hand, decreases its activity at pH lower than 7.45 while apparently maintaining its activity in the more alkaline range.

FIGURE VI  
CLOT RETRACTION IN CHICKEN BLOOD

Evidence of clot retraction in the blood of 10-week old broiler chickens and adult laying hens. The "cloudy" appearance of the hen serum is due to oestrogenic lipaemia.



### CLOT RETRACTION

A well marked clot retraction was observed in the blood of young chickens and laying hens. The studies on clot retraction, however, were made in the light of the following concepts:

1. Didisheim, Hattori and Lewis (Table III) indicated they found no clot retraction with chicken or duck blood.
2. The general agreement of many investigators, as indicated by Quick (52), that "the blood platelet is the primary cause of clot retraction."
3. The chicken does not have true "platelets" but has thrombocytes which are thought to be equivalent to platelets (Sturkie (61); Olson (48)).
4. Fenichel and Seegers (28) indicated that serotonin (5 hydroxytryptamine) from bovine platelets was one factor in bovine plasma clot retraction.
5. Quick (52) indicated an increase in the concentration of fibrinogen causes clot retraction to become less complete, also,
6. The amount of thrombin is a deciding factor in clot retraction.

Clot retraction was found to take place in the blood of both 10-week old chickens (broilers) and adult hens as illustrated by Figure VI. This clot retraction occurs more slowly than in the mammalian species as over 12 hours at water



bath temperature was necessary for complete clot retraction. Even after 24 hours at 42° C. the clot retraction in chickens was not as complete as that found in normal mammalian blood. The fact that it occurs in the absence of a true blood platelet, in blood with a high fibrinogen content, and in blood that apparently produces little thrombin, may be valuable in further general studies on clot retraction.

It will be noted in Figure VI that the blood plasma from the hens contains the "smoky" liquid material characteristic of lipaemia induced by natural oestrogens or the synthetic oestrogens hexestrol or dienestrol (Stamler and Warner (60)), but which has little effect in the clot retraction mechanism.



### NATURAL ANTICOAGULANT

The presence is indicated of one or more natural anticoagulants in avian blood, characterized by 1. extreme pH sensitivity, 2. anti-thrombic activity, 3. sensitivity to ionic strength, 4. adsorption on large amounts of tri-calcium phosphate, 5. precipitation with ammonium sulphate between 25 - 37% saturation, 6. presence in the chicken serum, 7. precipitation of one portion by heating at 65° C., 8. retention of another portion even after precipitation by heating at 100° C.

Evidence of an anticoagulant in chicken blood has been made by previous investigators: Seegers and Smith (58) noticed that chicken plasma inhibited the clotting of bovine fibrinogen with bovine thrombin and when mixed with bovine plasma lengthened the thrombin time from 15 seconds to 70 seconds. Stamler and Warner (60) found evidence of an anti-thrombic heparin-like substance in chicken plasma which varied in activity with oestrogenic concentration. Burstein and Guinand (14) found that chicken fibrinogen retarded the coagulation of human plasma.

In a number of experiments in this study, evidence of one or more substances having anticoagulant properties were found, which often interfered with test procedures as outlined for mammalian systems. These were:

Blood clotting times - As indicated by Table VI, the mean clotting time of whole blood with the addition of chicken



thrombin was 30.6 seconds, in contrast to 13.4 seconds with the addition of chicken thromboplastin. This may indicate the presence of a natural antithrombin in the chicken blood.

Thrombin production - A greatly prolonged clotting time upon recalcification of the euglobulin in the preparation of thrombin by the method of Biggs and Macfarlane (12) may have been due to an anticoagulant. The fact that clotting occurred more rapidly when the euglobulin was recalcified with double the dilution routinely used, and at 42° C. may indicate that dilution reduced the ionic strength and inhibited the effect of the anticoagulant. This is similar to the reversal of activity of anticoagulant fraction of incubated fibrinogen (AFIF) (64), (65).

Fibrinogen determination - In Table XII, two methods of obtaining a clot were used in the determination of fibrinogen from chicken plasma. The saline dilution method took a great deal longer to form a clot than did the distilled water dilution method. Also, the clot in saline had poor wrapping qualities resulting in lowered fibrinogen determination. This was similar in character to the AFIF of Triantaphyllopoulos (66), and may indicate an anticoagulant in chicken plasma. The reduction of the ionic strength by distilled water dilution was thought to inhibit the action of both anticoagulants, whereas if the ionic strength was maintained as in saline dilution, such inhibition did not come into full play.

pH Sensitivity - Although Seegers and Smith (58) indicate



that normal thrombin activity is sensitive to pH, the extreme sensitivity to pH of chicken plasma, as indicated in Figures II and III, is strikingly similar to the pH sensitivity as found with anticoagulant fraction of incubated fibrinogen (AFIF) by Triantaphyllopoulos (64).

Prothrombin determinations - In trials to find a satisfactory technique for prothrombin determination, rabbit adsorbed plasma was discarded as unsatisfactory, due to apparent species specificity. Chicken plasma was taken from an adult laying hen, adsorbed with 0.005 M. tricalcium phosphate and used to reconstitute a chicken prothrombin eluate. Duplicate reconstitution of the same eluate with rabbit adsorbed plasma gave a one-stage clotting time of 27 seconds with chicken thromboplastin, and 47 seconds with rabbit thromboplastin. The chicken adsorbed plasma reconstituted eluate gave a clotting time of over 600 seconds with rabbit thromboplastin and over 300 seconds with chicken thromboplastin. Adjustment of the pH of the chicken adsorbed reconstituted eluate with 6.2 pH imidazole buffer brought the one-stage clotting time down to 87 seconds. This would appear to indicate that an anticoagulant was present in the hen plasma that was not adsorbed with 0.005 M. tricalcium phosphate and was sensitive to pH changes. In later experiments, using 10-week old chicken plasma for prothrombin estimations, no such evidence of anticoagulant activity was apparent. This may tend to confirm the work of Stamler and Warner (60), wherein they found an

TABLE XVIII

THROMBIN TIMES OF ADSORBED CHICKEN PLASMA  
(SECONDS)

MOLARITY OF ADSORBING TRICALCIUM PHOSPHATE	UNADSORBED	.02	.04	.05	.055	.06	.065	.07	.075	.08	.1
Chicken plasma 1 *	33.5 (12.3)	22 (16.2)	19 (46)			13.5 (>900)				12.5 (>900)	11.5 (>900)
Chicken plasma 2 +	20.0 (11.8)			17.2 (82)	14.0 (3600)	13.4 (3600)	12.4 (3600)	12.0 (3600)	11.5 (3600)		

\* 0.2 ml. imidazole buffer pH 6.2  
0.2 ml. plasma  
0.1 ml. thrombin

( ) 1 stage prothrombin times in brackets

+ 0.1 ml. imidazole buffer pH 6.2  
0.2 ml. plasma  
0.1 ml. thrombin



TABLE XIX

THROMBIN TIMES<sup>+</sup> OF CHICKEN FIBRINOGEN  
AFTER ADDITION OF HEATED CHICKEN PLASMA \*

	PRECIPITATION	TIME FIRST STRANDS SEEN (sec.)	OBSERVATIONS	FINAL pH <sup>x</sup>
Saline Control	-	9.7	Rapid solid clot	
Unheated plasma	-	61.0	Only few flecks fibrin- Rest remained unclotted	7.64
Plasma heated to 45° C.	None	49.0	Only few flecks fibrin- Rest remained unclotted	7.42
50° C.	None	59.2	More clot evident - not solid up to 5 mins.	7.52
55° C.	Heavy	40.0	Clotting more rapidly- easier to see first fibrin strands and clot solid in 9.5 sec. after	7.5
60° C.	Heavy	38.9	First strands easily seen and clot solid in 10 sec. after	7.51
65° C.	Heavy	33.8	First strands easily seen- solid clot 7 sec. after	7.5
70° C.	Heavy	35.0	20 sec. for solid clot after first fibrin strands seen	7.5
75° C.	Extremely heavy	39.1	Good clot - 7 sec. after first strands	7.4
100° C.	Solid white clot	20.5	Good solid clot at first sign.	7.32

+ Mean of at least 4 experiments.

\* 0.1 ml. chicken thrombin + 0.2 ml. of mixture of 2 ml. heated or unheated plasma, 0.5 ml. chicken fibrinogen and 1.25 ml. pH 6.6 imidazole buffer.

<sup>x</sup> Clots frozen at end of experiment and pH determined after 3 days.

antithrombin present in the plasma of chickens with high oestrogen content.

In experiments on amounts of tricalcium phosphate necessary to completely adsorb prothrombin from chicken plasma; thrombin times were used in conjunction with the one-stage prothrombin times. As indicated by Table XVIII, the thrombin times of the adsorbed plasma became shorter as the one-stage prothrombin times increased. This could indicate that an antithrombin was being removed by adsorption on the tricalcium phosphate. The pH sensitivity of the anticoagulant was again portrayed when thrombin times were attempted on plasma adsorbed with 0.06 M. tricalcium phosphate. Without buffer, the thrombin times were 27 seconds; with 6.2 imidazole buffer, the thrombin times were 13.5 seconds.

Table XIX gives the result of thrombin times on buffered chicken fibrinogen mixed with chicken plasma that had been heated at varying temperatures. An equally heavy precipitation occurred between the 55° - 70° C. At 75° C. there was an extremely heavy precipitation, while at 100° C., the whole sample formed a solid white clot.

The presence in the plasma of something anticoagulant in nature, is indicated by the difference in time of 61 seconds for the unheated plasma, compared with 9.7 seconds with the saline control. Heating to 65° C. apparently removed some of the anticoagulant permitting a shorter clotting time of 33.8 seconds, and a more firm resultant clot, while heating

TABLE XX

## COMPARATIVE EFFECT OF THROMBIN AND THROMBOPLASTIN

ON CHICKEN FRACTION #2 AT 42° C.  
(SECONDS)

THROMBIN TIME *		ONE-STAGE PROTHROMBIN TIME <sup>x</sup>		
SALINE + FIBRINOGEN	FRACTION #2 + FIBRINOGEN	SALINE + PLASMA	FIBRINOGEN + PLASMA	FRACTION #2 + PLASMA
10.6	14.1	14.2	19.1	15

\* Mean of 8 thrombin experiments using:

0.1 ml. chicken fibrinogen and 0.1 ml. chicken thrombin with 0.1 ml. of either  
Fraction #2 or saline.<sup>x</sup> Mean of 5 thromboplastin experiments using:0.1 ml. of equal parts of fresh oxalated chicken with saline, chicken fibrinogen  
or Fraction #2.



FIGURE VII  
 COMPARISON OF BOVINE AND CHICKEN THROMBINS  
 WITH A.F.I.F., CHICKEN AND RABBIT PLASMA

	Bovine Thrombin	Chicken Thrombin
Chicken Plasma	> 150	20
Rabbit Plasma	5	10
A.F.I.F. + Rabbit Plasma	15	81

Clotting Time in Seconds  
 Comparison of Bovine and Chicken Thrombins

to 70° and 75° C. may have removed a procoagulant, resulting in another increase in clotting time to 35 and 39.1 seconds respectively. Evidence that not all anticoagulant was removed even at 100° C., is indicated by the 20.5 second clotting time with the heated chicken plasma, compared with the saline control of 9.7 seconds.

Since anticoagulant fraction of incubated fibrinogen (AFIF) is prepared from plasma with an ammonium sulphate saturation higher than 25% (63), a "fraction No. 2" was prepared from chicken plasma representing ammonium sulphate saturation between 25 and 37 percent. This material was tested by conducting thrombin times on chicken fibrinogen mixed with "fraction No. 2", and one-stage prothrombin times with fresh oxalated chicken plasma mixed with "fraction No. 2". The results given in Table XX reveal antithrombin activity by the prolongation of thrombin time from 10.6 seconds for the saline control to 14.1 seconds. This antithrombic effect was not seen with the one-stage prothrombin time tests.

Figure VII illustrates the action of AFIF in rabbit plasma on chicken thrombin and bovine thrombin, indicating that AFIF also competes readily for chicken thrombin. Table IX reveals a greatly prolonged clotting time of euglobulin when a small amount of chicken serum was added prior to recalcification. This would appear to indicate the presence of an anticoagulant in chicken serum either antithromboplastic or antithrombic in nature.



SPECIES SPECIFICITY

Specificity of blood coagulation materials by species was underscored by the relative inactivity of chicken blood with materials prepared from other animals.

The species specificity of blood coagulation materials has been noted by many authors. Seegers (57), Quick (51), and Mann and Hurn (46), emphasized the species specificity of thromboplastin. Quick (51) also pointed out the species variation of prothrombin. Seegers and Smith (58) indicated that antithrombin varied from species to species. Didisheim, Hattori and Lewis (25) noted little difference in mammalian thromboplastins or thrombins, but a decided variation between these and the avian materials.

Evidence of species specificity was also noted in these studies. Figure VII compares the action of bovine and chicken thrombin on chicken plasma and rabbit plasma. The clotting of the chicken plasma with bovine thrombin is greatly prolonged, however, chicken thrombin can clot rabbit plasma in only double the time of bovine thrombin. Table VIII indicates the one-stage prothrombin time of two fresh chicken plasmas was greatly prolonged when rabbit brain thromboplastin was used, in comparison with chicken brain thromboplastin. Table XVI emphasized that apparent species specificity of chicken prothrombin. This, giving a greatly prolonged clotting time when reconstituted with rabbit adsorbed plasma, even if rabbit thromboplastin were used.

TABLE XXI  
BLOOD YIELD ON EXSANGUINATION

SPECIES	WEIGHT (Kilo)	BLOOD YIELD (ml.)	YIELD PER KILO (ml.)
Chicken	3.75	135	36.0
Chicken	2.5	80	32.0
Duck	1.85	80	43.25
Pheasant	.84	40	47.5

MISCELLANEOUS OBSERVATIONS

A number of observations were made in the course of the avian coagulation studies that could not be included under separate headings, due to the small number of experiments. These are mentioned, as they may be worthy of further investigation.

BLOOD YIELD ON EXSANGUINATION:

Table XXI gives the yield of blood from the jugular vein upon complete exsanguination of three species.

SPONTANEOUS CLOTTING OF EUGLOBULIN:

Euglobulin prepared from citrated chicken plasma according to the method of Biggs and Macfarlane (12), but prior to recalcification, spontaneously clotted within six days of refrigeration storage at 4° C. This experiment was repeated using plasma from the same pool, but centrifuged at 2,000 r.p.m. and 10,000 r.p.m. respectively. Euglobulin prepared from these, failed to spontaneously clot after eight days refrigeration storage at 4° C. This could indicate that some coagulating factor had contaminated the first euglobulin bringing about a spontaneous coagulation. Such contamination may have been with a coagulase positive Staphylococcus organism.

FACTOR V:

Factor V is thought to be essential in the formation of thrombin with the tissue thromboplastin, and to be a factor in its formation with plasma thromboplastin (Figure I). In the thromboplastic activity test of cellular elements, as



indicated in Table IX, chicken serum gave a greatly prolonged clotting time of the euglobulin following recalcification. As Factor V is consumed upon clotting of blood, no Factor V is present in the serum, and its lack may explain this prolonged clotting time. In another series of experiments on thromboplastin activity, one plasma in six tests gave a mean recalcification time of 250 seconds. The same plasma was again tested after refrigeration storage at 4° C. for 7 days. At this time, in 5 tests it gave a mean clotting time of 1,200 seconds. This too, could indicate the reduction in some labile factor such as Factor V.

ACCELERATED CLOTTING FOLLOWING FREEZING OF PROTHROMBIN:

In preliminary testing of a satisfactory procedure for estimation of prothrombin by the adsorption and elution technique, chicken prothrombin was adsorbed on both 0.005 and 0.015 M. tricalcium phosphate solutions, eluted with 0.2 M. sodium citrate, and reconstituted with adsorbed rabbit plasma. The mean one-stage clotting time of prothrombin by both concentrations was 91 seconds, when tested on the day of adsorption. The concentrated sodium citrate eluate was frozen at -25° C. for 24 hours, aliquots again reconstituted with fresh adsorbed rabbit plasma, and one-stage prothrombin time determinations made. A mean of 10 readings for both eluates was now 48 seconds, almost half the reading of the day previous. It would appear that something in the freezing process had an effect on the prothrombin to shorten clotting

TABLE XXII

FACTOR VII INFLUENCE ON CHICKEN ELUATE  
 RECONSTITUTED WITH RABBIT ADSORBED PLASMA  
 (ONE-STAGE PROTHROMBIN TIME IN SECONDS)

RECONSTITUTED WITH	0.1 ml. eluate 0.9 ml. plasma	0.1 ml. eluate 0.1 ml. rabbit serum 0.8 ml. plasma	0.1 ml. saline 0.1 ml. serum 0.8 ml. plasma	0.1 ml. eluate 0.1 ml. human serum 0.8 ml. plasma	0.1 saline 0.1 human serum 0.8 plasma
Experiment 1	46.4 (8.8%)	13.9 (60-20= 40%) <sup>x</sup>	24.9 (20%)		
Experiment 2	51 (7.8%)			14.4 (59-10=48.1%) <sup>x</sup>	39 (10.9%)

\* Mean of 5 experiments

<sup>x</sup> Corrected by deducting prothrombin content of sera

% Prothrombin content in percentage of human from prothrombin table of Quick (52)

time. This is reminiscent of the conversion of prothrombinogen to prothrombin, as indicated by Quick (52).

FACTOR VII:

Factor VII is thought to be essential in the formation of thrombin with tissue thromboplastin (Figure I). In preliminary prothrombin determinations of chicken plasma by the adsorption and elution technique using reconstitution with rabbit adsorbed plasma, the addition of rabbit or human sera to provide Factor VII accelerated the prothrombin time, as shown in Table XXII. It would appear that Factor VII may not have been adsorbed from the chicken plasma with the concentration of tricalcium phosphate used, or was not present initially.



DISCUSSION

It would appear from Table I that previous investigators had been classifying the clotting time of chicken blood in the range of mammals, except for Delezenne (23), Howell (37), (38), (39), and Seegers (57), who noted that the clotting time of chicken blood was "slow", and Stamler and Warner (60) who found a prolonged clotting time in the blood of laying or oestrogen treated chickens. All were using methods of blood collection that would include varying amounts of tissue thromboplastin. Even the siliconized needle technique used here must include some tissue thromboplastin in varying quantities, as indicated by the different clotting times noted in the various tubes. There is the possibility that if chicken blood could be collected without the contamination with tissue thromboplastin, that the time of blood coagulation may be surprisingly prolonged. This would present the opportunity of observing only the action of plasma thromboplastin.

Knowledge of the prolonged blood clotting time, in the absence of tissue thromboplastin, could be used in collecting chicken blood from the wing veins to avoid deaths of valuable birds, such as occasionally occurs in cardiac puncture collection.

If the blood of a mammal clotted as slowly as that of the hens tested, one could expect a severe hemorrhagic diathesis, however, aside from pathological conditions, the laying chicken has an efficient blood clotting mechanism. This apparently

FIGURE VIII

COMPARISON OF CLOTTING TIME OF WHOLE BLOOD  
WITH AND WITHOUT ADDED THROMBOPLASTIN OR THROMBIN

COAGULATION TIME AT 42° C

CHICKEN BLOOD WITH AND WITHOUT ADDED THROMBOPLASTIN OR THROMBIN

NUMBER of BIRDS	MATERIAL ADDED	CLOTTING TIME (SECONDS)	CONTROL LEE-WHITE CLOTTING TIME (MINUTES)
21	CHICKEN THROMBOPLASTIN	13·4 (11 — 17·8)	70
15	CHICKEN THROMBIN	30·6 (19 — 50)	64·5

relies on an active tissue thromboplastin, rather than plasma thromboplastin as does the mammal.

According to the generally accepted schema of blood coagulation (Figure I), the addition of preformed chicken thrombin to whole blood should have brought about a more rapid clotting than the addition of thromboplastin, which could only assist in the formation of thrombin within the blood sample. Such was not the case, as indicated by Figure VIII, the thrombin addition clotting in a mean time of 30.6 seconds, whereas the thromboplastin addition took only 13.4 seconds. No apparent explanation is evident. Two possibilities present themselves: 1. the chicken thrombin added was not present in quantities as large as that formed as the result of thromboplastin addition, 2. an antithrombin in the blood may have delayed the action of the preformed thrombin.

The finding that clotting was only slightly longer at 37° C. than at 42° C., may be more apparent than real. It is felt that since the clotting mechanism relies upon the enzymatic action of thrombin, and that such enzymatic actions generally are accelerated by increased temperatures, that further work may indicate a longer clotting time at 37° C.

It is possible that further work with other ages and sexes of chickens may reveal differences in clotting time, prothrombin and fibrinogen content due to the fluctuation in blood constituents, as demonstrated by the following authors: Gonzaga (34) found a variation in many blood constituents



in chickens from one day to eight months of age. Tidrick, Joyce and Smith (62), utilizing the two-stage method of Warner, Brinkhous and Smith (68, 69) for prothrombin determinations, indicate that day-old chicks have only 30% the amount of prothrombin of the adult, and that the prothrombin content in adults' blood is 20% higher in summer than in winter. Stamler and Warner (60) found a prolonged clotting time in hens and oestrogen treated birds, due to the presence of an antithrombin.

In the light of the long clotting time of blood from hens and the variations found in clotting times apparently due to contamination with natural tissue thromboplastin; the variations in blood clotting times of previous investigators (Table I) are understandable and may even cast some doubt on the validity of the conclusions reached by Johnson and Conner (42) in their work on avian lymphomatosis, and Warnock, Clarkson and Stevenson (70) in their studies of atherosclerosis in cholesterol fed cockerals. Fortunately, in the original work on Vitamin K by Dam, Schonheyder, Almquist, Stokstad and Quick (1, 2, 4, 5, 22, 27, 50, 55), a tissue thromboplastin in some form was used which would reduce errors due to natural tissue thromboplastin contamination, but would not interfere with the prolongation of clotting due to a deficiency in prothrombin formation. Long clotting time, extreme pH sensitivity, the presence of anticoagulant activity and species specificity, however, could have been interfering with the



results of the following investigators who used avian coagulation as a tool: Cover, Mellen and Gill (19), Glover and Humble (40), Cravens, Randle, Elvehjm and Halpin (20), Hare, Anderson, Weakley, Bletner (36), Barnett et al (8, 9), Gray et al (35), Bornstein and Samberg (13), Asplin and Boyland (7) and Stamler and Warner (60).

The fact that three different breeds of hens were used in the clotting tests and that their blood clotted rapidly upon the addition of chicken thromboplastin or thrombin, would tend to preclude errors due to Vitamin K deficiency, congenital or other defects in their avian blood clotting mechanism. There appears to be evidence to indicate that chickens are deficient in some blood coagulation factors that are found necessary in the mammal (Wartelle (71, 72); Soulier, Wartelle and Menach (59); Didisheim, Hattori and Lewis (25))., but the lack of factors essential to the mammalian system do not necessarily indicate that the avian blood coagulation mechanism is abnormal.

No clotting activity was present in a thromboplastin prepared from chicken jugular veins, even though an active brain thromboplastin can be prepared and evidence indicates that a strong "in vivo" thromboplastin is present in the chicken. It was observed, during the preparation of the vein thromboplastin, that a great deal of fine material was decanted off with the used acetone. There is a possibility that the thromboplastic activity of the jugular veins may be



confined mainly to the endothelial lining, the cells of which may have been initially removed with the first changes of acetone in the dehydration process. Other tissues do possess thromboplastic activity, as indicated by Table II, various authors using embryo, muscle or lung extracts satisfactorily. A practical application of the thromboplastic activity of traumatized tissue was the "squeezing" of the wound, following venipuncture to halt bleeding in donor chickens which previously had continued for some minutes. Application to poultry surgery may be possible.

The slight differences in plasma clotting time with chicken brain thromboplastin mixed with 5 ml. of saline at the rate of 0.2 grams and 0.15 grams (Table VIII) would appear to indicate an adequate excess of thromboplastic activity in chicken brain thromboplastin, although interference with this activity by mixing with distilled water is also shown.

The role of the avian thrombocyte in blood clotting needs investigation. Thromboplastic activity appeared evident in the cellular elements of chicken blood containing thrombocytes or fragments of thrombocytes. The hemostatic mechanism of the chicken, however, in the light of the prolonged clotting times found, cannot rely upon the formation of plasma thromboplastin of which the thrombocyte should be a major component. Overcoming the difficulties in segregation of the thrombocytes from other blood cellular elements, necessary to detect this role, may be based on the fractionation methods of Bessis (11).



In the light of the high prothrombin content of chicken plasma, the very low yield of chicken thrombin is surprising. One would think that the thrombin yield should parallel the content of prothrombin. This does not appear to be the case. Three possible explanations may be advanced:

1. chicken fibrinogen adsorbs or combines with chicken thrombin more readily than does the human fibrinogen with human thrombin, thus decreasing the amount of free thrombin left in solution,
2. our techniques of fibrin removal may not be satisfactory for complete thrombin yield,
3. the possibility of an anti-thrombin being present which would greatly reduce the amount of thrombin activity left in a "thrombin" preparation.

The fact that a euglobulin clot was lysed by incubation overnight at 42° C. would indicate the presence of fibrinolytic activity. This may have been the thrombin itself (43), or some other fibrinolysin.

The variation in fibrinogen readings between the distilled water and saline dilution methods and the difficulty of obtaining a clot that was completely removable, could be responsible for many errors in quantitative determination. The modified method described is felt to be reliable.

Prothrombin determinations by the several methods used, revealed great variability, but the one-stage technique appeared to be the most accurate. The amounts found by the adsorption and elution technique should have compared with these, but the presence of anticoagulants and the amounts of tricalcium phosphate must be overcome before accurate comparison



can be made.

The prolongation in chicken plasma clotting times noted with variations in pH, may explain some of the difficulties encountered by previous investigators, e.g., Schonheyder (55) encountered chicken plasma clotting times in the range of 120 to 600 seconds. He also observed that "chicken plasma clots more and more slowly with each day of standing"; the prolonged clotting of chicken plasma noted by Carel and Ebeling (17) that necessitated setting their fibroblast tissue cultures to one side for varying periods of time to ensure clotting; Quick (50) who indicated that he had difficulty obtaining chicken brain thromboplastin of a uniform activity.

The fact that clot retraction does take place in the chicken blood, indicates that some cells, probably the thrombocytes, are taking place in the retraction mechanism. The fact that clot retraction occurs slowly may be due in part to the high fibrinogen content or to poor conversion of prothrombin to thrombin, as was found with thrombin preparation, or even a combination of these with the large amount of fibrin adsorbing all the thrombin generated. The thrombocyte or other cell that is engaged in clot retraction too, may be more resistant to thrombin than are mammalian platelets, thus not taking part as rapidly in the release of enzyme or chemical causing the shrinking of the fibrin strands.

Species specificity, as indicated by the prolonged clotting time of chicken materials or plasma with those of the



mammalian species, emphasized the difference between avian and mammalian blood coagulation mechanisms.

The presence of one or more anticoagulants in avian blood would explain several phenomena encountered in these studies and by other investigators. Prolonged thrombin time, sensitivity to changes in pH and ionic strength are also characteristics of the antithrombin AFIF (anticoagulant fraction of incubated fibrinogen) as shown by Triantaphyllopoulos (63,64,65,66) and a like material, very similar to fibrinogen may be part of the anticoagulant seen here and observed by Burstein and Guinand (14). One portion of anticoagulant was precipitated by heating to 65° C. (Table XIX), but anticoagulant activity still persisted in the blood serum left after precipitation of protein by heating to 100° C.

The heparin-like antithrombin found by Stamler and Warner (60) in laying hens and oestrogen treated chickens may have been a factor in some observations, but the majority of the test procedures indicating anticoagulant activity, were conducted with plasma from 10-week old chickens of mixed sexes, precluding it as the only anticoagulant factor.

The evidence that one or more of the anticoagulants may be adsorbed on large quantities of tricalcium phosphate may prove a useful tool in their further study and separation.

Future experiments using chicken blood coagulation may avoid questionable results if the characteristics of prolonged clotting time, pH sensitivity, species specificity



and evidence of natural anticoagulants are taken into consideration.



CONCLUSIONS

1. The blood of 37 caged laying hens of three different breeds had a mean clotting time of 69.25 minutes, indicating the generation of very little plasma thromboplastin and reliance for efficient blood clotting upon an active tissue thromboplastin.

2. Acetone dehydrated chicken brain thromboplastin (52) was an active coagulation material, but thromboplastin prepared in a like manner from chicken jugular veins, was unsatisfactory.

3. The mixture of chicken brain thromboplastin with distilled water instead of saline gives a prolongation of plasma clotting time of up to 1.8 seconds.

4. Chicken thrombin prepared by the method of Biggs and Macfarlane (12) had only 1/20 the activity of human thrombin produced in the same way.

5. Chicken thrombin could not be extracted following euglobulin clot lysis.

6. The presence of a fibrinolysin in chicken plasma is demonstrated.

7. The quantitative estimation of fibrinogen in plasma from chickens 10 weeks of age was 355 mgs. of fibrinogen per 100 ml. of plasma, compared with the normal for humans of 250 to 400 mgs. (Quick (52)).

8. The prothrombin content of plasma for 10-week old chickens was 136.6% of human prothrombin levels by the

CHAPTER 1

The first chapter of the book is devoted to the study of the properties of the function  $f(x)$  defined on the interval  $[0, 1]$  by the formula  $f(x) = x^2 \sin \frac{1}{x}$ . It is shown that this function is continuous on the whole interval  $[0, 1]$  and that it has a unique maximum at  $x = \frac{1}{\sqrt{2}}$ . The proof of these facts is given in detail. In the second chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied more thoroughly. It is shown that the function is differentiable at every point of the interval  $(0, 1]$  and that its derivative is  $f'(x) = 2x \sin \frac{1}{x} - \cos \frac{1}{x}$ . The function  $f'(x)$  is not bounded on the interval  $(0, 1]$ , but it is continuous at every point of this interval. In the third chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the origin. It is shown that the function oscillates infinitely often as  $x \rightarrow 0$ , but that the amplitude of these oscillations tends to zero. In the fourth chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = 1$ . It is shown that the function is differentiable at  $x = 1$  and that its derivative is  $f'(1) = 2 \sin 1 - \cos 1$ . In the fifth chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = 0$ . It is shown that the function is not differentiable at  $x = 0$  and that its derivative does not exist at this point. In the sixth chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = \frac{1}{\sqrt{2}}$ . It is shown that the function has a unique maximum at this point and that its derivative is zero at this point. In the seventh chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = \frac{1}{\sqrt{2}}$ . It is shown that the function is differentiable at this point and that its derivative is zero at this point. In the eighth chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = \frac{1}{\sqrt{2}}$ . It is shown that the function is differentiable at this point and that its derivative is zero at this point. In the ninth chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = \frac{1}{\sqrt{2}}$ . It is shown that the function is differentiable at this point and that its derivative is zero at this point. In the tenth chapter the properties of the function  $f(x) = x^2 \sin \frac{1}{x}$  are studied from the point of view of its behavior near the point  $x = \frac{1}{\sqrt{2}}$ . It is shown that the function is differentiable at this point and that its derivative is zero at this point.

one-stage technique. The adsorption and elution techniques yielded lower figures due to species specificity, presence of an anticoagulant or incomplete adsorption of prothrombin.

9. The mean pH of fresh whole chicken blood determined by an open cup technique was 7.535 which gradually became more alkaline upon exposure to air, presumably by loss of carbon dioxide. Oxalated chicken plasma was found to have a mean pH of 7.9 (range 7.8 - 8.0). Native plasma removed from blood cells or in contact with blood cells had a similar pH.

10. Both chicken thrombin and chicken thromboplastin appear to have an optimum activity at approximately pH 7.45. Thrombin activity is reduced above this, whereas thromboplastin activity is reduced below this point.

11. Clot retraction did occur in coagulated chicken blood but was not as complete as in mammalian blood.

12. Species specificity precludes the use of mammalian coagulation materials in avian clotting studies.

13. Modifications to mammalian blood coagulation techniques needed for application to chicken plasma included:

1. a siliconized needle technique of venipuncture for more accurate blood clotting determinations,

2. increased temperature, plasma dilution, and calcium content in thrombin production and fibrinogen determinations,

3. adjustment of pH with acidic buffers when conducting clotting times for consistent results,



4. distilled water dilution only for satisfactory "wrappable" clots in fibrinogen determinations.

14. One or more natural anticoagulants were found in chicken blood which greatly influenced its behavior in coagulation studies. The anticoagulants exhibit the characteristics of: antithrombin activity, sensitivity to variations in pH and ionic strength, ability to be adsorbed on tricalcium phosphate and precipitation of one portion by heating to 65° C., but retention of another portion in the serum after removal of protein by heating to 100° C.

15. If avian blood coagulation is to be used as a tool in future investigations, consideration of the characteristics of chicken blood found in these studies will avoid questionable results. These characteristics are:

1. long clotting time,
2. slow generation of plasma thromboplastin and reliance on tissue thromboplastin,
3. pH sensitivity,
4. species specificity,
5. evidence of one or more natural anticoagulants.



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